An Immunomodulatory, Tissue Deposition Mechanism and Rehabilitative Therapy for Restoring Muscle Functionality After Volumetric Muscle Loss

by

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Volumetric Muscle Loss is a condition that affects military and general population suffering from significant skeletal muscle trauma that leads to 20% or more skeletal muscle loss. Volumetric muscle loss (VML) is a significant clinical burden that results in long-term functional deficits such as chronic pain and loss of muscle strength and mobility, as well as fibrosis, fatty infiltration, and inflammation. Standard care for VML includes autologous muscle flap transfer followed by exercise rehabilitation. This procedure can result in complications such as immune rejection, insufficient vascularization, fibrosis, contractures, and poor innervation. Furthermore, rehabilitation-mediated functional improvements after VML are limited. A dysregulated immune response after VML injury has been shown to impair myogenesis and exacerbate fibrosis, ultimately leading to muscle wasting. These effects are partially mediated through Transforming Growth Factor β1 (TGF-β1) overexpression by a dysregulated macrophage population. TGF-β1 has been linked to several muscle pathologies that result in fibrotic accumulation such as ALS and muscular dystrophy. Specialized pro resolving-lipid mediators (SPM) are produced by

essential fatty acids and are signaling molecules that resolve inflammation and have shown to be promising treatments for immune system disorders. We have previously investigated the SPM Resolvin D1 (RvD1) for its ability to attenuate pro-fibrotic signaling and enhance muscle regeneration by resolving chronic inflammation in the VML injury niche. Our overall hypothesis is that local delivery of a stable isomer of RvD1, Aspirin- Triggered RvD1 (AT-RvD1), will foster a pro-regenerative microenvironment in the VML injury to enhance engraftment of a tissue-engineered muscle construct for improved functional muscle regeneration. Additionally, we hypothesize exercise rehabilitation will further enhance functional muscle recovery.

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Introduction

Volumetric muscle loss is a clinical burden that affects both military and civilian populations and results in long-term functional deficits including loss of mobility, fibrosis, fatty infiltration, and inflammation [1]. The standard care for volumetric muscle loss is an autologous muscle flap procedure. The autologous muscle flap procedure in total aims to prioritize revascularization and reinnervation of muscle tissue to mitigate amputation and muscle atrophy of the affected area of significant skeletal muscle trauma. Unfortunately, this procedure is accompanied by functional deficits of the muscle such as loss in muscle strength due to common post-operative complications of immune rejection, donor sight morbidity and insufficient vascularization [2].

Researchers have implemented tissue-engineering strategies to promote muscle regeneration *in vivo*, however these strategies are limited by the lack in the ability to address chronic inflammation. The dysregulated immune response resulting from the VML traumatic injury can be linked to, but not limited to, TGF-β1 which has been shown to impair myogenesis and exacerbate fibrosis. To take a wholistic view of treatment for VML, we have investigated three objectives of treatment. The first objective, we address the dysregulated immune response through investigating a SPM candidate AT-RvD1 that can attenuate TGF-β1 effects *in vitro*. The second objective, an *in vivo* method for tissue deposition uses an aligned collagen scaffold to meet the demands of muscle regeneration unmet by natural physiological response to VML. The third objective is the investigation of enhancing muscle functionality throughout the treatment period through regenerative rehabilitation regimen by way of voluntary

wheel running *in vivo*.

All three forms of treatment were investigated separately to better understand the extent to which each treatment individually can address the dysregulated immune response of the VML injury that causes long term functional deficits in patients suffering from VML.

AT-RvD1 in vitro investigation: AT-RvD1 is aspirin triggered RvD1. When RvD1 is aspirin triggered it contributes to a more potent anti-inflammatory effect [23]. AT-RvD1 has been shown to downregulate TGF-β1 signaling. It is critical to harness a greater capacity of AT- RvD1s promising ability to resolve inflammation and downregulate TGF-β1 in the VML injury. It is the objective to investigate myotube formation in response to a range of TGF-β1 ***d***osages (0.5 ng/mL, 1 ng/mL, 2.5 ng/mL). In addition, it is the objective to investigate myotube formation in response to co-treatment of TGF-β1 ***d***osage range with 100 ng/mL of AT-RvD1 as a counteracting agent to inhibit anti-myogenic behaviors of TGF-β1. We hypothesize that the primary myoblasts dosed with 2.5 ng/mL of TGF-β1will reduce myogenesis, but these effects will be attenuated by the application of AT-RvD1 treatment and restore myogenesis in the anti- myogenic environment. With the use of immunohistochemistry staining techniques, we analyzed myosin heavy chain II expression and quantify myotube formation to identify a healthy myogenic environment.

Myoblast deposition in vivo via aligned collagen scaffold: Our objective is to investigate the extent to which myoblast deposition is successful in restoring muscle functionality for a VML injury using an aligned collage scaffold for myoblast delivery to a critical sized defect of 3mm to the quadriceps in a mouse model. To evaluate the

functional outcomes of myoblast deposition *in vivo* via aligned collagen scaffold, there was the implementation of isometric torque analysis at the end of the treatment course to evaluate the isometric contractile strength of the injured muscle. We hypothesized that the mice receiving a deposition of myoblasts via an aligned collagen scaffold will have increased muscle functionality relative to mice receiving a blank aligned collagen scaffold. The isometric torque analysis is used for measuring isometric contractile muscle strength relative to a femoral nerve stimulus of the quadricep. Increases in torque contraction can demonstrate that transplantation with myoblast deposition by way of an aligned collagen scaffolds is a promising method in restoring muscle functionality after VML.

Exercise Rehabilitation Regimen in vivo: Rehabilitation is a keystone treatment regimen in orthopedic injury models to enhance the injured musculoskeletal unit during the duration treatment to restore strength, mobility, and resilience of the musculoskeletal unit [24]. We have implemented voluntary wheel running as the rehabilitation component in the treatment of the VML mouse model**.** The objective of this investigation is to evaluate voluntary wheel running as a modality for rehabilitation by quantifying the extent to which voluntary wheel running restores muscle functionality during the duration of the treatment period post VML injury. It is our objective to investigate the rehabilitation of sub-critical 2 mm defect and critical 3mm defects in the mouse quadriceps for the VML mouse model [3][4]. Evaluating a rehabilitation regimen against a critical and sub-critical sized defect allows for us to evaluate the regenerative capacity of the rehabilitation on two defect types for greater comparison. Mice undergoing rehabilitation regimen receive running wheels at 1-week

post-injury and undergo pre training 2 weeks prior to operation. Wheel running is re-implemented post operation and recovery. We hypothesized that there would be a significant increase in torque muscle contraction for rehabilitation groups in both the critical and subcritical sized defects.

Findings: From our investigation of AT-RvD1 invitro we found that AT-RvD1 attenuates TGF-β1 inhibition of C2C12 and Primary Myoblasts cell myogenic differentiation.

Our myoblast deposition method via an aligned collagen scaffold *in vivo* showed that muscle stem cell transplantation alone does not lead to muscle functionality and recovery. Exercise rehabilitation regimen *in vivo* showed promising trends towards restoring muscle functionality, but there is more opportunity for investigation for harnessing the full potential of an exercise rehabilitation regimen with a co-treatment intervention to address the dysregulated immune response at the site of the injury.

There is little investigation into the co-treatment of VML with pro-resolving mediators with the clinically used paradigm of tissue transfer and exercise rehabilitation. Our overall future objective after conducting three investigations on treatment methods addressing the functional deficits in gold standard VML treatment, is to improve functional muscle regeneration in VML- injured muscle using a novel therapy that synergizes tissue-engineered muscle constructs with immunomodulatory SPMs and rehabilitation exercise.

Background

Volumetric Muscle Loss

VML is a clinical burden as it is established to be a rapid bulk muscle loss condition that can overwhelm the capacity for surgical repair. The Extremity Trauma and Regenerative Medicine, U.S. Army Institute of Surgical Research (2015) identified the orthopedic cohort as medically retired servicemembers who sustained a combat-related type III open tibia fracture. Of this cohort, 65% of the disability of the patients is defined by a muscle condition. Among the general cohort, defined by the battlefield-injured service members who are medically retired, 92% of the muscle conditions were defined as VML [22]. VML often results in long term deficits including loss of mobility, fibrosis, fatty infiltration, and inflammation. These deficits can lead to lifelong dysfunction and disability which can contribute to an overall altered whole- body metabolic rate and respiratory exchange ratio due to loss of physical activity with loss of muscle functionality [13]. The clinical gold standard treatment for VML is the autologous muscle flap procedure. The autologous muscle graft procedure involves the transfer of an autologous muscle flap from an undamaged muscle to the injury site. Unfortunately, this treatment can result in functional deficits of the muscle as this procedure can often result in graft necrosis, donor site morbidity and insufficient vascularization [25]. Further concern for VML includes the dysregulated immune response after VML injury as we have previously shown that it can impair myogenesis and exacerbate fibrosis, ultimately leading to muscle wasting.

The accumulation of fibrotic tissue because of long-term deficits of VML is caused by a dysregulated immune response that prevents the body from transitioning

to a pro-regenerative state [5]. The dysregulated immune response can be characterized by a variety of systems; however, this thesis focuses on the dysregulation of the cytokine transforming growth factor beta 1 (TGF- β1). We and others have previously found the gene for the pro-fibrotic growth factor TGF- β1 to be significantly elevated in an aberrant population of fibro-adipogenic progenitor (FAP) cells associated with fibrotic outcomes after VML by Shannon Anderson, a previous lab member of the Willett Lab in her thesis on aberrant cellular response and muscle regeneration following volumetric muscle loss. This data found that there was elevated secretion of TGF- β1 in the VML injury due to elevated expression of the profibrotic gene beta1- integrin, the population of FAPs that express high levels of adhesion protein B1-H1. In non-elevated levels of TGF-β1 this growth factor regulated cellular functions such as cell growth, differentiation, and apoptosis and is found abundantly in bone and cartilage. However, pathologically high levels of TGF- β1 can drive fibrosis in the muscle after VML. TGF-β1 promotes a pro-fibrotic state by causing fibroblasts to transform into myofibroblasts that produce large amounts of collagen in addition to the stimulation of ECM proteins with the inhibition of their degradation. The promotion of the pro-fibrotic state of the muscle after VML also contributes to promoting fibroblast differentiation and overall inhibition on myogenesis of skeletal muscle [11].

The VML injury model in the murine model of the quadriceps was developed by Brian M. Sicari, Vineet Agrawal, Bernard F. Siu, Christopher J. Medberry, Christopher L. Dearth, Neill J. Turner, and Stephen F. Badylak. The researchers were affiliated with the McGowan Institute for Regenerative Medicine and the Department of Pathology at

the University of Pittsburgh. The VML injury model in the mice quadriceps requires the use of a biopsy punch which creates a thickness defect that can be characterized as critical sized or sub critical sized defect. A 3mm biopsy punch removes about 15% of the quadriceps muscle mass which creates a critical-sized defect. The critical sized muscle defect results in the overall irrecoverable loss of muscle. The irrecoverable loss of muscle is characterized by the incomplete bridging of myofibers followed by persistent inflammation and fibrosis. It is established that after 56 days the critical-sized defect heals with a remaining dense collagenous scar tissue [15].

Signaling Pathways of TGF-β1 and AT-RvD1

Skeletal muscle cell differentiation is a multistep process that involves cell cycle withdrawal that leads to alignment and fusion of myoblasts to form multinucleated muscle fibers. Skeletal muscle differentiation is controlled by a complex set of trophic and growth factors that take part in coordinating and responding to environmental cues for myoblasts to differentiate and mature into fused muscle fibers. Transforming Growth Factor- β1 is a trophic factor produced in the body naturally. TGF- β1 at normal physiological levels is crucial for immune regulation, tissue repair and more [18]. However, overexpression of TGF-β1 inhibits the myogenesis in skeletal muscle by reducing cell fusion, reducing satellite cell proliferation, repressing muscle- specific genes, and inducing fibrotic cells [6]. TGF-β1 signaling pathway inhabits skeletal muscle differentiation and growth through the repression of MyoD which is an umbrella of transcription factors [5].

The TGF-β1 ligand binds to both type I and type II receptors threonine/serine kinases on the cell surface of skeletal muscle [19]. The signal from TGF-β1 ligan

binding promotes the signaling pathway through which the phosphorylation of Smad proteins occurs. Smad3 represses the activity of MyoD and interferes with MyoD/E protein heterodimerization [5]. MyoD is a critical regulator in skeletal muscle differentiation. A treatment modality to regulate upregulated TGF-β1 includes, but is not limited to, a pro resolving lipid mediator (SPM) such as AT-RvD1 that has shown to have immunomodulatory capabilities for anti-inflammatory application in bronchial epithelial cells, macrophage regulation in immunoregulation and other pathophysiological applications [9][20].

AT-RvD1 is a lipid mediator that has anti-inflammatory and pro-resolution effects. Its production is initiated by resolution of inflammation and is derived from the omega 3 fatty acid docosahexaenoic acid. The body can increase RvD1 levels through the consumption of Eicosapentaenoic acid and docosahexaenoic acid along with aspirin, however the body does not produce enough RvD1 for the capacity of repair required for VML [10]. As the body does not naturally produce enough RvD1 to attenuate a targeted response to TGF-β1 and other upregulated growth factors during the dysregulated immune response, there is a critical need to investigate the immunomodulatory potential of delivering AT-RvD1 exogenously in the VML model [12]. For this reason, investigating the tunability of AT-RvD1 attenuating properties of upregulated TGF-β1 can provide promising steps towards a AT-RvD1 delivery mechanism for direct treatment to the site of injury in VML.

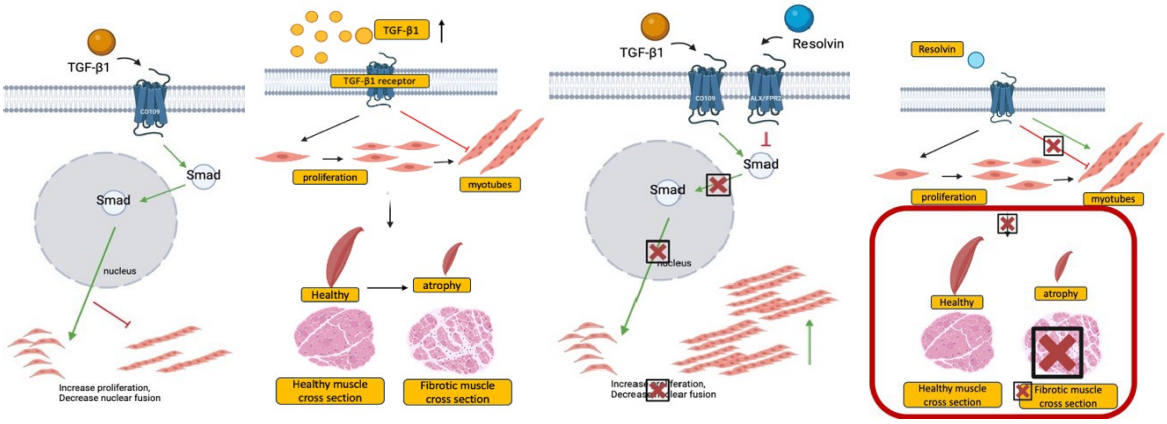

Figure 1: **Signaling Pathways of TGF-β1 and At-RvD1** (left two panels) TGF- β1 signaling pathway in skeletal muscle in inhibition of myogenesis with upregulated TGF- β1. (right two panels) Resolvin signaling pathway, attenuation of TGF-β1 inhibition of myogenesis pathway. Biorender Schematic.

Myoblast Delivery and Rehabilitation

A structurally aligned myoblast-seeded scaffold technology is a promising material used for cell delivery with the objective of improving functional muscle recovery. Previous studies have shown that using aligned matrices is successful in improving functional muscle regeneration and vascularization *in vivo* following VML. Aligned myoblast seeded scaffolds provide promising benefits for muscle regeneration [7]. The scaffold technology can provide contact guidance which can direct myoblast growth [8]. Maturation and development of longer myofibers can be improved which generates a higher fusion index. Other forms of treatment investigated for combating muscle wasting and muscle functionality loss are wheel running regimens in the case of a VML injury model in mice.

Rehabilitation regimen, also known as wheel running exercise in mice is used as a mode of assessment and promotion of physical performance and overall endurance. Wheel running is established as a voluntary activity that is in contrast with other experimental exercise models in mice. Voluntary wheel running involves the individual housed mice to receive slanted, plastic, saucer-shaped wheels individually in the cages. The rotations of the wheel are electronically recorded and quantified for running rate and frequency. The benefits of voluntary wheel running in the mice model are the overall reduction of the negative effects of stress on the mice and increase in the promotion of resilience to chronic stress.

Overall understood running patterns in mice include that mice tend to increase their running activity in the first three weeks with a plateau after the overall increase. Wheel running activity in the presence of an injury model may show differing trends compared to uninjured mice [16].

Methods

AT-RvD1 invitro investigation

Objective: It is the ***objective*** to investigate myotube formation in response to a range of TGF-β1 dosages (0.5 ng/mL, 1 ng/mL, 2.5 ng/mL) to identify if there is a dosage response in the muscle cells to TGF-β1. It is the objective to also investigating myotube formation in response to co-treatment of TGF- β1 dosage range with 100 ng/mL of AT-RvD1 treatment.

Hypothesis: We ***hypothesize*** that the primary myoblasts treated with 2.5 ng/mL of TGF- β1 and AT-RvD1 will show greater myotube formation per biological replicate compared to primary myoblasts treated with only 2.5 ng/mL of TGF- β1. The types of results used to analyze the *invitro* model includes immunohistochemistry staining and imaging. There are seven stages of preparation required to analyze the potential attenuating effects of AT-RvD1 on TGF- β1 anti- myogenic effects.

Experimental Design and Timeline: Stage 1 requires the coating of a T75 flask and 24 Well Plate with collagen and laminin to increase cell adhesion, growth, and viability. Stage 2 involves the seeding of primary myoblasts on T75 flask and 24 well plate. Stage 3 involves the expanding of primary myoblast cells to ~60/70% confluency in the T75 flask and grow cells to confluency in the 24 well plate at ~60/70% confluency. Stage 4 involves the passaging and freezing of the T75 flask sample of cells at ~60/70% confluency that are not used for the duration of the experiment and using the remaining cells to implement into treatment groups to 24 well plate.

The cells are then grown to ~60/70% confluency to implement treatment at the correct window of growth needed to quantify myotube formation against the treatment groups received. See Fig. 2. For in-depth schematic of each treatment group.

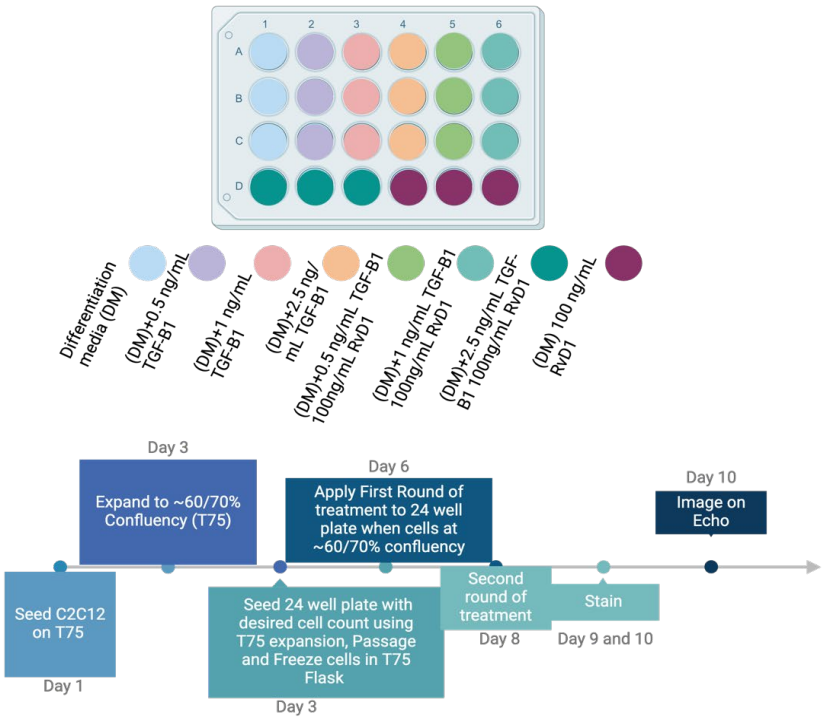

Figure 2: **2D Cell Culture Schematic for *In vitro* Investigation of TGF-B1 response to AT-RvD1 treatment**. *In vitro* investigation of TGF-β1 attenuation using the pro-resolving lipid mediator AT-RvD1 as a treatment. Biorender Schematic.

Parameters for each treatment include the following ratios. **TGF- β1 Working Solution:** 7.5 µL TGF-B1 and 30 µL Differentiation Media, **Untreated:** 500 µL Differentiation Media per well, **0.5 ng/mL TGF- β1:** 498.5 µL Differentiation Media + 0.5 µL of TGF-B1 Working Solution + 1 µL of 200 proof ethanol per well, **1 ng/mL TGF- β1:** 498 µL Differentiation Media + 1 µL of TGF-B1 Working Solution + 1 µL of 200 proof ethanol per well, **2.5 ng/mL TGF-β1:** 497.5 µL Differentiation Media + 2.5 µL of TGF-B1 Working Solution + 1 µL of 200 proof ethanol per well, **0.5 ng/mL TGF- β1+100 ng/mL RvD1:** 498.5 µL Differentiation Media + 0.5 µL of TGF-B1 Working Solution + 1 µL of 100 ng/mL AT-RvD1 per well, **1 ng/mL TGF-**

β1+100 ng/mL RvD1: 498 µL Differentiation Media + 1 µL of TGF-B1 Working Solution + 1 µL of 100 ng/mL AT-RvD1 per well, **2.5 ng/mL TGF- β1+100 ng/mL RvD1:** 497.5 µL Differentiation Media + 2.5 µL of TGF-B1 Working Solution + 1 µL of 100 ng/mL AT-RvD1 per well, **100 ng/mL RvD1:** 499 µL Differentiation Media + 1 µL of 100 ng/mL AT-RvD1 per well.

Stage 5 involves the application of the second round of treatment to 24 well plate. Cells are replenished with media during this second round to provide fresh nutrients to the cells. Stage 6 involves staining. To quantify myotube formation it is vital to use immunohistochemistry and Leica thunder imaging for quantification analysis. Quantification of myotube formation begins with the fixing of the primary myoblasts. Primary myoblasts are fixed in the fume hood using 4% paraformaldehyde once cells have differentiated into myotubes and are at a set confluency, that if surpassed would lead to overgrowth.

Following the fixing of the primary myoblasts, there is the application of permeabilization buffer to enhance interactions of the primary antibodies with cytoplasmic and nuclear target proteins in fixed samples. Following the permeabilization buffer there is the application of the blocking buffer. The blocking buffer is used to reduce non-specific binding that can obstruct true signals leading to inaccurate interpretations. Following the application of the blocking buffer there is the application of the primary antibody staining which targets the myosin heavy chain II protein present in myotubes.

Following the application of the primary antibody staining there is the application of DAPI which targets the adenine and thymine rich regions of nuclei of cells for fluorescent application. Furthermore, with the DAPI application there is the additional application of the secondary antibody staining allowing for the fluorescence of myotubes visible by TxRed.

Stage 7 involves imaging using the ECHO and Leica Thunder imaging systems. Both imaging systems allow for the capturing of the fluorescence of the nuclei and myotubes allowing for the quantification of the myotube formation. Imaging involves taking 3 sample images per well. Once all sample images are taken the images are ready for processing. Using the images in either the ECHO or the Leica Thunder imaging system you can quantify myotube formation by counting the number of myotubes per image. Furthermore, the analysis involves identifying the fusion index. The fusion index is calculated by the number of nuclei inside the myosin heavy chain II-positive myotubes divided by the total number of nuclei present in a field of view.

The C2C12 cells provide the opportunity to examine our treatments on an immortal cell line. Once tuned, the treatment groups can then be investigated with a more specific cell line applicable for myoblast deposition such as the primary myoblasts that are isolated from mice.

Prior to expanding the primary myoblast sample, the tissue culture plates need to be tuned to allow for optimal attachment for increased cell viability environment, as primary lines often lack the ability to adhere to a plastic surface. This tuned habitable environment is achieved using a collagen and laminin coating. The collagen and

laminin coating involves the dilution of collagen to 50 μg/mL with 0.01-0.02 HCl. This solution is added to 10μg/cm^2 onto the surface of each tissue culture surface required. In this case, we used 2 T75 flasks and one 24 well plate for the process of the collagen and laminin coating to expand our initial sample of primary myoblasts prior to culturing in a 2D tissue culture plate.

The surface is properly coated and allowed to incubate for 2 hours. A wash is implemented using PBS to remove any excess acid which did not evaporate.

The laminin coating involves the slow thawing of the laminin to avoid gelation. Once properly thawed, the laminin is diluted in HBSS. The solution was applied to the desired plates and immediately after application was aspirated for greatest use and cell adhesion.

The treatment groups involved in the TGF-β1 dosage response using C2C12 cells were replicated in primary myoblasts. Furthermore, staining and imaging analysis for C2C12 cell were also implemented for the analysis of the primary myoblast *in vitro*.

Myoblast deposition in vivo via an aligned collagen scaffold

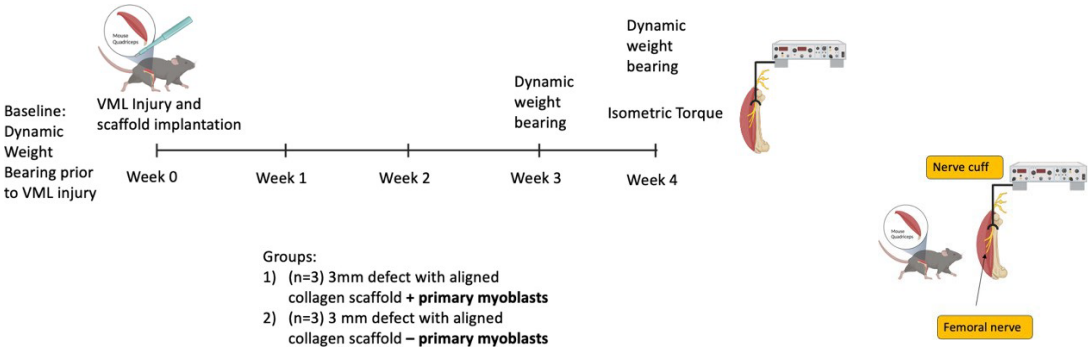

Figure 3: **Timeline of myoblast deposition via aligned collagen scaffold in murine model for a VML injury** Biorender Schematic.

Objective: It was our **objective** to investigate the impact of seeded (with primary myoblasts) collagen scaffolds on increased muscle functionality post volumetric muscle loss defect.

Hypothesis: We **hypothesized** that the 8–12-week-old male C57/BL6 mice injured with a full-thickness, 3mm quadriceps VML defect would generate greater isometric torque relative to animals treated with blank collagen scaffold. Primary myoblasts were used to investigate the viability of the collagen scaffolds in a VML model for skeletal muscle delivery.

Experimental Design and timeline: ***2-Day Hydration of Collagen-Based Scaffolds.*** A total of 12, 3 mm size defect collagen scaffolds were individually placed into a 6-well plate for storage. A 100% sterile-filtered ethanol is added to each well for sterilization of the scaffold for promoting cell viability in the scaffold environment. The plates were placed on a shaker in an incubator at 37ºC for 2 hours. The ethanol it was aspirated with a Pasteur pipette for the application of 6 mL of sterile PBS addition to the wells to wash out the ethanol. The well plates were placed on a shaker in an

incubator at 37ºC for 15 minutes. The PBS was aspirated and repeated for additional wash of the scaffold material. Appropriate mass of EDAC/NHS crosslinker was measured out for crosslinking the collagen scaffolds. This crosslinker was implemented to promote cell adhesion and migration onto the scaffold. The crosslinking solution was then added to each well and the plates were placed on a shaker in an incubator at 37ºC for 1.5 hours. To each well 6 mL of PBS was added after the aspiration of the crosslinking solution and plates were placed on a shaker in the incubator at 37ºC for 15 minutes.

This process was then repeated but the time in the incubator was increased to 45 minutes. Once the PBS was aspirated, 6 mL of proliferation media was added to each well and placed on a shaker in an incubator at 37ºC overnight. The following day, the media was aspirated. New 6 mL media was added to each well. The plate was then put on a shaker in an incubator at 37 ºC overnight for 48 hours of soaking in the media. The next day the scaffolds were prepped for myoblast seeding.

Primary Myoblast Seeding on Collagen-Based Scaffolds. Once the scaffolds have successfully soaked in proliferation media overnight the scaffolds are prepared to be seeded with primary myoblasts. The old media was removed from each well and each scaffold was then placed in an Ultra-low attachment 6-well plates. An ultra-low attachment plate is used to reduce cell growth on other surfaces other than the scaffold. Primary myoblasts were counted and added to each side of the scaffold. Prior to applying primary myoblasts to the other side of the scaffold, the first application remained parallel to the well plate surface for 15 munities to allow for adhesion. The plates were then returned to the incubator for 2 hours to allow for cell attachment. 5-6

mL of proliferation media was then added to each well.

The media is changed every 3 days over the course of the experiment. To induce differentiation, differentiation media is applied once cells have reached ~60% confluency. Liquid media itself is a liquid nutrient diet for cells.

Differentiation media encourages cells to specialize through low serum content, and growth media promotes cell proliferation through high serum content.

Operational protocols for mice surgeries in VML: VML Surgery, Pre- and Post- Operative Care. Pre-Operation involves the induction of the rodent at 5% (oxygen at 1) when defect is being made. When the animal is fully under, the nose cone is transferred and the isopropyl is lowered to 2% (oxygen stays at 1). The eye lubricant and Buprenorphine SR (23G needles) are applied. The rodent is flipped so its stomach is facing up to shave the area required to visualize the defect site. The leg is wiped with isopropanol and chlorohexidine for three rounds for sterilization of the defect site field. As the area is sterile, the rodent is transferred to the operation room to undergo a defect to the quadriceps using a 3 mm biopsy punch. During the duration of the operation, the animal is checked 10-15 minutes for vitals and constitution via a leg pinch and visualizing responses to oxygen and isopropyl. Once the rodent is cleared to move to post-operative care based on the completion of the operation, the rodent is transferred to the post-operative space.

The skin of the rodent is tented to administer the 2 mLs of lactated Ringer's to enhance the inflammatory response post operation. Hydrogen peroxide is applied to a small piece of gauze to gently wipe any blood on the skin. Iodine is applied to the incision followed by metronidazole applied to the wound clips to reduce irritation. The animal is weighed to allow for the monitoring of weight during the duration of the study. In the cage there is adequate fuel and enrichment for the rodent for the duration of the rodent's post-operative care.

Exercise Rehabilitation Regimen In vivo

Objective: To investigate the impact of exercise in a sub-critical and critical defect (2 mm versus 3 mm) in the quadricep and use muscle strength testing to evaluate the functional outcomes using isometric torque analysis.

Hypothesis: We hypothesized that mice receiving voluntary wheel running would display greater isometric torque relative to groups receiving no rehabilitation. Furthermore, we hypothesize groups receiving a critical defect would have decreased isometric torque relative to mice receiving a subcritical sized defect in groups receiving voluntary wheel running groups.

Experimental Design and Timeline: 12 mice received a VML defect (6 with 2 mm defect vs 6 with 3 mm defect). The mice received one week of acclimation prior to surgeries and running wheels were introduced to Group 1 (2 mm defect) and Group 3 (3 mm defect) on Day 7 post-surgery. Muscle functionality was tested at the end of week 4 of treatment to evaluate muscle functionality via isometric contractile quantification through isometric torque measurements by way of quadriceps stimulation through stimulation of the femoral nerve. See *Operational protocols for mice surgeries in VML* for pre and post operation of VML defect workflow.

Animal Group	Number of Animals	Running Wheel
Group 1 (2 mm defect)	3 mice	With running wheels
Group 2 (2 mm defect)	3 mice	Without running wheels
Group 3 (3 mm defect)	3 mice	With running wheels
Group 4 (3 mm defect)	3 mice	Without Running wheels

Quantitative measurement: Running wheels, half of each group received a running wheel to analyze the absence or presence of muscle strength restoration. Running was introduced at day 7. We measured the isometric force produced by a contraction after we stimulated a pulse when we cuff the femoral nerve. Isometric torque production by knee extensor muscles was measured at endpoints. The femoral nerve was isolated and stimulated using a nerve cuff with pulse duration, frequency, and train duration (0.5 ms, 175 Hz, and 500 ms) to elicit isometric tetanic torque Pulse Duration: 0.2 ms, pulse interval: 5.7 ms (Frequency 175 Hz) to quantify muscle strength.

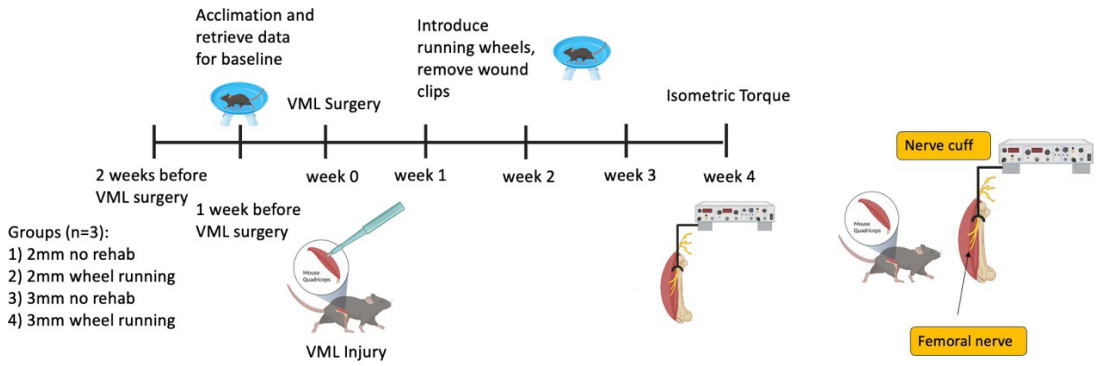

Figure 4: **Timeline of exercise rehabilitation regimen implementation in murine model for a VML injury.** GAIT measurements were taken during the study but are not currently processed. Biorender Schematic.

Results

In vitro: AT-RvD1 attenuates TGF- β1 inhibition of C2C12 cell myogenic differentiation

Myosin heavy chain expression of differentiated C2C12 cells and primary myoblasts were identified by way of immunohistochemistry staining (**Figures 5 and 6**). This staining allowed for the fluorescence of both myosin heavy chain isoform II via TxRed fluorescing a red color and nuclei via DAPI fluorescing a blue color. Images of the stained C2C12 and primary myoblast cells were taken on a Leica Thunder fluorescent microscope at 4x and 10x magnification. Each treatment group was composed of 3 biological replicates. Within each biological replicate, there were three images analyzed for an individual technical replicate.

Within each image, we looked to capture the proliferated and differentiated cells. Proliferated cells that are undifferentiated do not fluoresce the standard TxRed, rather their signal is only identified by the DAPI staining indicating presence of nuclei. Differentiated cells fluoresce both the TxRed and blue color, indicating myosin heavy chain expression in a differentiated myoblast forming a myotube. Using ImageJ macros, the relative fluorescence intensity was calculated for each image (**Figure 5**). Using GraphPad we conducted a one-way ANOVA for statistical comparison of all treatment groups.

A statistically significant difference was found between the control (differentiation media, DM) and 1 ng/mL TGF-β1+ differentiation media in addition to a statistically significant difference between the control and 2.5 ng/mL TGF-β1+ differentiation media in myosin heavy chain expression (**Figure 5**). Less myosin heavy chain expression was observed in cells treated with increasing dosages of TGF-

β1. When introducing RvD1 as a treatment, we found a statistically significant difference between 2.5 ng/mL TGF-β1 and 2.5 ng/mL TGF-β1 + differentiation media + Resolvin 100 ng/mL. Greater myosin heavy chain expression was observed in cells treated with Resolvin in the presence of TGF-β1.

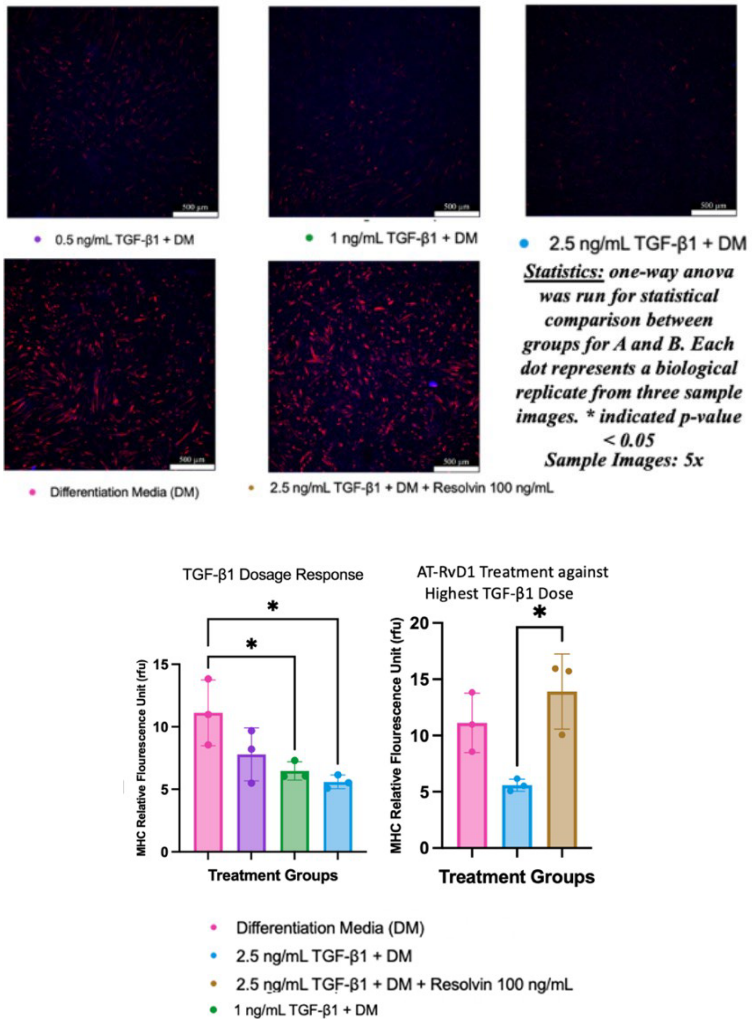

Figure 5: **TGF-β1 response to AT-RvD1 in C2C12 cells**. Images (4x) of C2C12 myotubes stained using immunohistochemistry with a primary and secondary antibody (primary: anti-Mouse myosin heavy chain II, secondary: Goat anti-rabbi Ig H&L alexa flour 594). Row one from left to right are increasing dosages of TGF- β1. Row two from left to right displays highest dose of TGF-β1 with countering treatment of AT-RvD1 for TGF- β1 attenuation. Graphs display the myosin heavy chain II expression as it relates to each treatment group.

In vitro: AT-RvD1 attenuates TGF-B1 inhibition of primary myoblasts myogenic differentiation

When examining the same treatment groups applied to primary myoblasts, we found a statistically significant difference between the control (differentiation media) and 1 ng/mL TGF- β1 in addition to a statistically significant difference between the control and 2.5 ng/mL TGF-β1 (**Figure 6**). However, when examining 2.5 ng/mL TGF-β1 + differentiation media + Resolvin 100 ng/mL treatment vs 2.5 ng/mL TGF-β1 + differentiation media there was no statistical significance found in average myotube count. Moreover, there was no significant difference found between 2.5 ng/mL TGF-β1 + differentiation media + Resolvin 100 ng/mL treatment and our control treatment differentiation media in average myotube count. When examining nuclear fusion in primary myoblasts treated with 2.5 ng/mL TGF-β1 + differentiation media + Resolvin 100 ng/mL vs 2.5 ng/mL TGF-β1 + differentiation media we found a statistically significant difference in nuclear fusion. Myotubes in cells treated with 2.5 ng/mL TGF-β1 + differentiation media + Resolvin 100 ng/mL had more nuclei per myotube relative to 2.5 ng/mL TGF-β1 + Differentiation media, which had fewer nuclei per myotube (**Figure 6**).

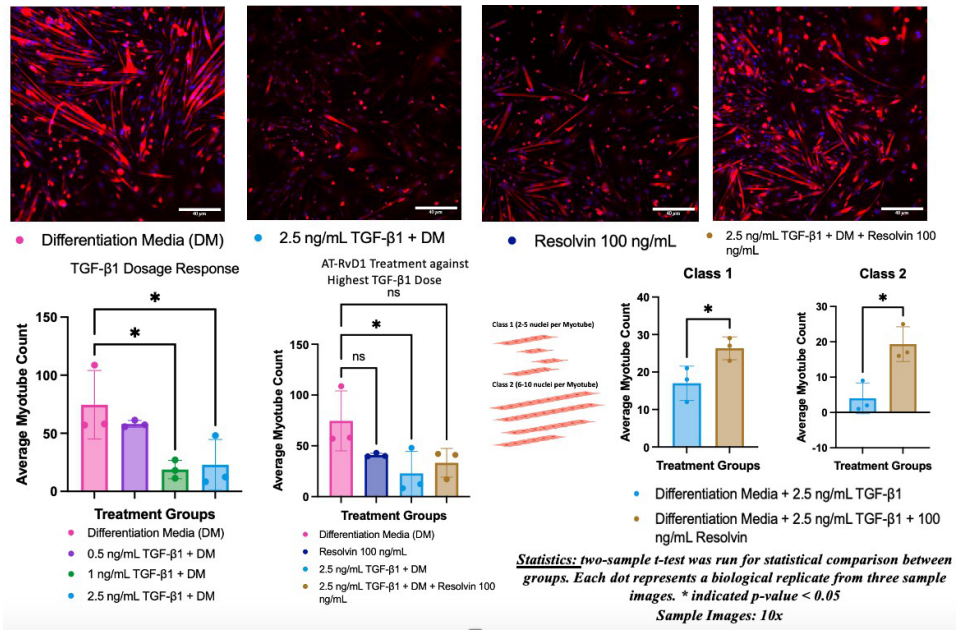

Figure 6: **TGF-β1 response to AT-RvD1 in primary myoblast cells**. Images (10x) of primary myoblast myotubes stained using immunohistochemistry with a primary and secondary antibody (primary: anti-Mouse myosin heavy chain II, secondary: Goat anti-rabbi Ig H&L alexa flour 594). Graphs on the left display the average myotube count respective to each treatment group. Graphs on the right display average myotube count respective to each treatment group respective to the highest dose of TGF- β1 and class of myotube.

<u>In vivo: Decreased functional outcomes in injured limb receiving collagen scaffold with exogenous myoblast delivery</u>

When examining isometric torque values for *in vivo* work on myoblast deposition via a collagen scaffold, we used a two-sample t-test for statistical (**Figure 7**). When comparing all contralateral (control) isometric torque values to all injured isometric torque values, statistical significance was found between the two groups. Mice contralateral (control) quadriceps displayed greater isometric torque values than mice quadriceps treated with myoblast deposition via a collagen scaffold. To establish %contralateral, the isometric torque values of both treatment groups were normalized to the contralateral isometric torque values. When examining the %contralateral recovery of muscle isometric torque between two treatment groups, seeded collagen scaffold and blank collagen scaffold, no significant difference was found. Further, no significant difference was found between the two treatment groups in un-normalized comparison.

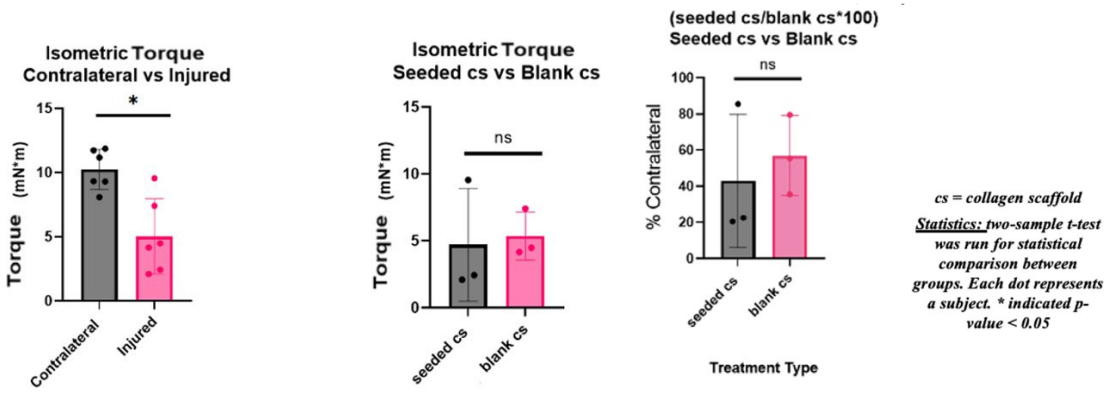

Figure 7: **Isometric torque analysis of myoblast deposition via aligned collagen scaffold.** Graph 1 measures the isometric toque contralateral vs the injured limb measured in torque (mN*m). Graph 2 represents Seeded cs vs Blank collagen scaffold treatment measured by % contralateral. Graph 3 measures isometric torque seeded collagen scaffold vs blank collagen scaffold measured in torque (mN*m). Number of mice in pilot study (n=6).

In vivo: Rehabilitation significantly increases sub-critically sized defects muscle strength

When examining isometric torque values for *in vivo* work for voluntary wheel running, we used a two-sample t-test for statistical analysis (**Figure 8**). When examining the normalized maximum torque values, mice in the 2 mm defect group receiving voluntary wheel running had statistically significant isometric torque values relative to sedentary mice in the 2 mm defect group ($p < 0.05$). Voluntary wheel running in 2 mm defect resulted in increased isometric torque ($p < 0.05$). When examining the 3 mm defect and introducing wheel running, we observed trends toward improvements in isometric torque, but we did not have a large enough sample size in our sedentary group to see a significant result. When examining the differences between isometric torque values in voluntary wheel running mice, in both 2 mm defect groups and 3 mm defect groups, the 3 mm defect groups were significantly behind the 2 mm group in isometric torque values ($p < 0.001$).

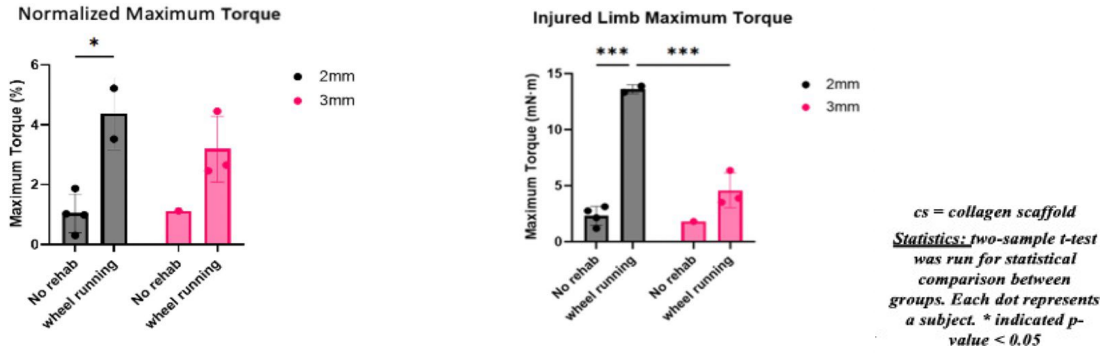

Figure 8: **Isometric torque analysis of rehabilitation regimen treatment, voluntary wheel running.** Graph 1 measures the normalized maximum torque in analysis of Maximum Torque (%) in mice receiving no rehab vs wheel running. Graph 2 measures Injured limb maximum torque (mN*m) in mice receiving no rehab vs wheel running.
Number of mice in pilot study (n=12)

Discussion

The current gold standard treatment to address VML's rapid muscle loss does not account for the dysregulated immune response present at the site of VML injury. This results in chronic pathophysiology's such as chronic inflammation that can lead to fibrosis, muscle wasting and atrophy with overall loss in muscle strength. The use of the autologous muscle flap as a gold standard treatment in patients suffering from a VML injury allows for the mitigation of muscle limb amputation reduction but does not account for muscle wasting and targeting the restoration of muscle functionality. To account for this gap in knowledge, restoring muscle functionality and reducing in muscle wasting, we implemented i*n vitro* and *in vivo* studies to investigate novel rehabilitation regimens and immuno-regenerative therapies in the VML injury model. We hypothesized that AT-RVD1 could foster a pro-regenerative microenvironment invitro in the presence of TGF-β1. We further hypothesized that myoblast deposition would address rapid muscle loss and result in increased muscle strength after VML injury. From a rehabilitative standpoint we hypothesized rehabilitation in the form of voluntary wheel running would enhance muscle regeneration and increase muscle strength.

The effects of TGF-β1 (an anti-myogenic and pro-fibrotic condition) and the pro- resolving lipid mediator, AT-RvD1, were investigated in both primary and C2C12 cell line myoblasts. Immunofluorescent imaging from the cell culture studies showed that AT-RvD1 increased myotube formation in C2C12 cells and in primary myoblasts when cultured in anti- myogenic and pro-fibrotic conditions (Fig. 1A **p=0.0061, 1A *p=0.0264, 1B **p=0.0088).

Myoblasts were cultured on collagen sponges where the cells proliferated, differentiated, and fused to form MHC expressing myotubes. Cell seeded collagen scaffolds were implanted into a murine of quadriceps VML critical defect. In isometric torque analysis at week 4, decreased muscle strength was observed when comparing the average isometric torque of the injured limb receiving cell-laden collagen scaffolds compared to the average isometric torque of the contralateral. Further, exercise rehabilitation in the form of voluntary wheel running moderately improve strength outcomes as measured by isometric torque analysis about the knee at week 4.

Limitations to the investigation of AT-RvD1 attenuation of TGF-β1's anti-myogenic effects include the ability to identify from an intracellular signaling pathway standpoint when and where AT-RvD1 treatment leads to TGF-β1 attenuation. Further investigation into the signaling pathway of TGF-β1 in C2C12 and primary myoblasts will need to be investigated for a greater understanding of at what point in the signaling pathway TGF-β1 is responding to AT- RvD1 treatment. It is widely accepted in literature that RvD1 suppresses TGF-β1 through the activation of the ALX/FPR2 and GPR32 receptors. RvD1 activates FPR2, which inhibits TGF- β1through Smad signaling. As a result, our work was able to display attenuation of TGF- β1 effects through AT-RvD1 treatment. Possible avenues to further investigate the signaling pathway involving the timing and location of AT-RvD1 attenuation of TGF- β1 effects would involve fluorescence microscopy and immunohistochemistry to visualize the interaction between AT-RvD1. Further visualization of ALX/FPR2 receptor in addition to visualizing the regulatory affects AT-RvD1 has on the Smad cascade

would be important for AT-RvD1 characterization.

Limitations in the *in vivo* study of the deposition of myoblasts via an aligned collagen scaffold include not measuring for fibrotic conditions, sample size and a lack of more extensive negative and positive control groups. While the pilot study allowed for the opportunity to investigate the collagen scaffold treatment model, it had a small sample size which reduced statistical power. Conducting the pilot study promoted the ability to become familiar with the workflow required for an animal study, an essential skill to have in the field of bioengineering research.

Limitations in the *in vivo* pilot study investigating the implementation of a rehabilitation regimen in the form of voluntary wheel running include the inability to perform isometric torque measurements during multiple time points of the treatment period for greater comparison to takedown measurements. During the functional testing of the mice during isometric torque implementation, the nerve cuff used to stimulate the femoral nerve were inconsistent in size and functionality. As a result, the reproducibility of placing the nerve cuff and thereby the electrical stimulation to generate a contraction of the quadriceps muscle was not consistent.

In vitro: AT-RvD1 improves myogenesis in C2C12s and Primary Myoblasts. *In vivo*: Myoblast deposition via an aligned collagen scaffold and rehabilitation exercise showed modest improvements alone and may require combination treatment and co-delivery of immunomodulatory SPM to address the dysregulated immune response after VML injury.

Future directions include seeking to improve functional muscle regeneration using a novel therapy that synergizes tissue engineered muscle constructs with

immunomodulatory SPMs and rehabilitation exercise. Before incorporating all three areas of research of this thesis into one study that synergizes the individual treatments, it is important to characterize AT-RvD1 in addition to evaluate delivery methods of AT-RvD1 for the greatest immunoregulatory regulation.

Understanding the effects of AT-RvD1 on immune crosstalk with muscle stem cells would allow for a more in-depth characterization of myogenesis of AT-RvD1 treatment in myotubes. Avenues to explore for SPM delivery for targeted delivery *in vivo* includes using a hydrogel PEG-4MAL technology. PEG-4MAL is a synthetic hydrogel system composed of a four-armed polyethylene glycol macromer with associated maleimide groups. PEG-4MAL technology has been primarily applied to drug delivery and regenerative medicine research [17]. Using hydrogel PEG-4MAL technology will allow for controlled release and delivery of AT- RvD1. PEG-4MAL technology has been applied to a range of pathologies including muscle related acute injuries such as muscle tears. However, in the case of a VML injury there is the critical need to restore muscle volume and functionality which drives the need for deeper investigation into PEG-4MAL technology application for a co-delivery system: differentiated myoblasts and the pro resolving lipid mediator AT-RvD1 [14]. Application of a rehabilitation regimen is still applicable in the case of PEG-4MAL application, as the co-treatment method may be hindered by a sedentary group. Incorporating rehabilitation whether through voluntary wheel running or other supported exercise stimulus, it is important to mitigate loss of muscle strength during the treatment program for greater functional outcomes.

In all, our wholistic approach to treating VML has led us to identify both *in vitro* and *in vivo* findings that point towards the opportunity to synergize immunomodulatory regulation, myoblast deposition and voluntary wheel running rehabilitation. Our findings suggest AT-RvD1 improves myogenesis in C2C12 and Primary Myoblast cells and Myoblast deposition and rehabilitation exercise display promising trends in improving muscle strength in a VML injury.

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