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Effects of IL-10 On Local Cell Populations and Functional Recovery Following VML Injury

Zain Blackwell

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University of Arkansas Honors College

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Abstract

Volumetric muscle loss (VML) injuries are prevalent in both military personnel suffering from battlefield related incidents, and civilians following severe motor accidents. Despite its prevalence, VML has no pro-regenerative clinical treatments in place to recover some of the functional capabilities of the damaged muscle. Free flap grafting, debridement of damaged tissue, and physical therapy are the only clinical standards available that offer little functional recovery benefits, even after years of consistent treatment. In this study, anti-inflammatory cytokine interleukin-10 in conjunction with autologous minced muscle was assessed as a possible treatment for VML injuries and its influences on cellular behavior within the wound site.

1. Introduction

1.1 Significance

The prevalence of volumetric muscle loss (VML) injuries in military personnel suffering from battlefield related wounds and civilian motor vehicle accidents is astounding compared to the lack of standards of care for treating such injuries. Between 2001-2013 around 14,500 military personnel were medically evacuated from conflicts overseas (1, 2). Approximately 11,165 of those injuries were consistent with the mechanisms of VML (3). Because civilian related VML injuries are not as easy to track, the incident number of open fractures is commonly used to assess the prevalence of VML injuries in the civilian population. Between 3.5-6 million bone fractures occur annually in the US. Around 3%, or 150,000, of those fractures may be open fractures (4).

The only available standards of care to treat a VML injury are free flap grafting of skeletal muscle, surgical debridement of the damaged tissue, and physical therapy. These methods are generally ineffective and have no regenerative capabilities at all. Free flap grafting does not significantly improve contractile force recovery and is subject to high levels of fibrosis infiltrating the tissue (5). Another study found no functional improvements after external fixator and split thickness skin grafting, and a complete loss of dorsal flexion even after 1.5 years since injury (6). A 19-year-old war veteran showed no significant improvements after 3 years of physical therapy treatment targeting his vastus medialis muscle. It was not until surgical insertion of a multi-layered scaffold of extracellular matrix (ECM) and further physical therapy did functional recovery benefits reveal themselves (7). A 2014 study involving VML injured mice which prides itself on being the "first [to] demonstrate improvement in functional performance of non-repaired VML injured muscle with physical rehabilitation in the form of voluntary wheel running.", showed a 17% improvement in maximal isometric torque, and a 13% increase in weight of the injured muscle after weeks of voluntary wheel running (8). While these are hopeful statistics, it assumes those with leg injuries are able to run on the affected leg. This is typically unlikely, therefore making the parameters of a treatable population too narrow for it to be a generally effective treatment. After examination of these clinical treatments, there is a clear need to develop a treatment that could effectively manipulate factors of the body's innate repair system to recruit new muscle fibers to the wound site, or emulate the properties of the damaged tissue such as skeletal muscle, nerve cells, and satellite cells to eventually replace them.

The pathways involved in VML repair are still not fully understood, so it is equally important to have a thorough grasp on the repair system itself before entirely committing to the incorporation of clinical based therapies. The focus of this thesis both explores the efficacy of

injecting the anti-inflammatory cytokine interleukin-10 (IL-10) as a VML treatment, and its effect on macrophage polarization, T-cell aggregation, and progenitor cell recruitment and proliferation.

1.2 The VML Pathway

Skeletal muscle is able to regenerate its functional capabilities following minor injuries. A common example of this is working out. However, it is important to note that the recovery and improvement of muscle function is a result of hypertrophy, the increase in muscle fiber size, not the recruitment of new muscle fibers. In response to more substantial injuries, such as a VML injury, skeletal muscle function is irreversibly hindered without treatment. Damage to skeletal muscle is considered a volumetric muscle loss injury when a minimum of 20% of the relative muscle volume is damaged beyond repair or removed (9). VML repair can be broken down into four distinct phases: degeneration, inflammation, regeneration, and remodeling/repair.

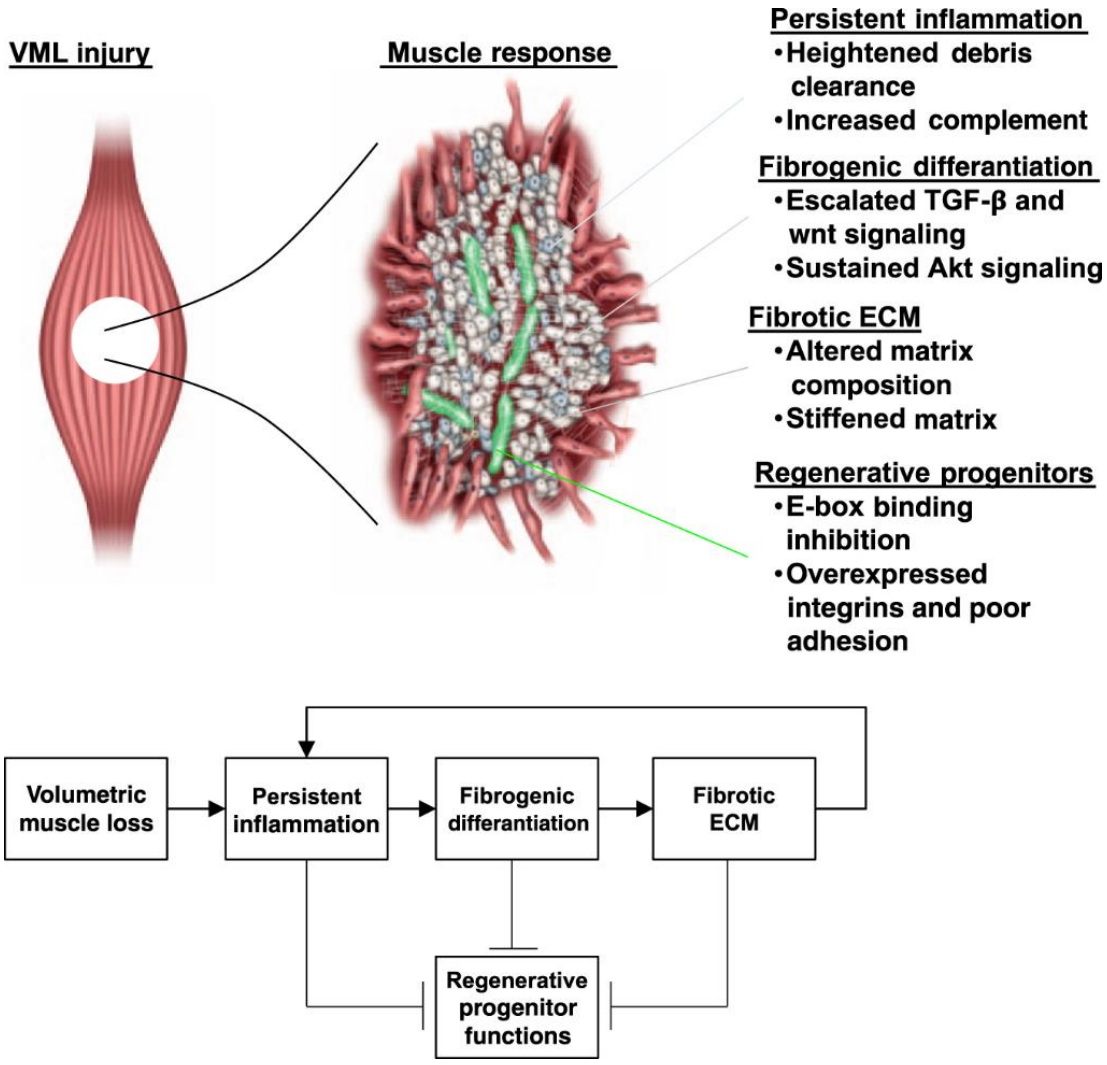

Figure 1: Representation of the four phases of a VML injury and the pathways involved. Ownership of this diagram belongs to: https://www.nature.com/articles/s41420-018-0027-8/figures/6

The degeneration phase is characterized by the necrosis of the damaged myofibers which then stimulates the inflammation stage where resident inflammatory cells recruit additional inflammatory cells such as neutrophils and macrophages to clear necrotic and foreign debris from the injury site (10). T-cells, also known as regulatory T-cells (Tregs) are recruited to promote M1 and M2 macrophage polarization and satellite cell activation (11). During the regeneration phage satellite cells, mesangioblasts, mesenchymal stem cells, and pericytes,

differentiate into myoblasts to replace the damaged muscle tissues, while less desirable fibroblasts may also infiltrate the damage site as scar tissue (10, 12). Satellite cells are the local myoblast precursors composing between 2-6% of the local myonuclei and exist between the basal lamina and sarcolemma. Because they are the primary stem cells that exist in the muscle region, they are influenced more by the cell to cell signaling that occurs during VML repair and are of particular interest in VML studies (13). Finally, in the remodeling/repair phase, the ECM is remodeled to represent its original state, angiogenesis occurs, and peak functional recovery may be observed (14).

1.3 Interleukin-10, M1 and M2 Macrophages, T-Cells, and Satellite Cells

The relationships between IL-10, macrophage polarization, and Tregs are all connected. They each play on each other while also each mediating the wound healing response. IL-10 is an anti-inflammatory cytokine secreted from Tregs primarily responsible for regulating inflammatory pathways. In the context of a VML injury, IL-10 seems to be downregulated vs its abundance in regular injuries and may partially contribute to the stunted regeneration of untreated VML injuries. Its leading effect is its inhibition of the production of pro-inflammatory cytokines. IL-1, IL-6, and interferon (IFN)-γ are a few of the cytokines affected (15). It has other suppressive roles, but they were not the focus of this thesis. Instead, more was explored into IL-10's functional recovery benefits and how these benefits are mediated through its effects on local cell populations (16, 17).

The occurrence of macrophage polarization is a milestone event in the transition from the inflammation stage to the regeneration stage of VML repair. M1 macrophages function to clear the injury site of foreign and necrotic debris by releasing the pro-inflammatory cytokines IL-1, and IL-6 in addition to pro-inflammatory cytokines IL-8, IL-12, and tumor necrosis factor (TNF)

(18). Like most things, a moderate presence of these cytokines is tolerable, and necessary for the facilitation of an inflammatory response, but the prolonged, unregulated presence of these cytokines contribute to chronic pain. In the case for unchecked accumulation of TNF, it may contribute an inflammatory response containing symptoms similar to septic shock and multi-organ failure (19). M1 macrophages are the largest producer of these cytokines compared to other cells at the wound site, so IL-10 is an important aspect in ensuring the timely transition from the inflammatory stage to the regeneration stage and mitigation of cytotoxic behavior. M2 macrophages, are much more desirable for the facilitation of the regeneration stage. M2 macrophages promote cell proliferation, ECM remodeling, and have been shown to induce angiogenesis (14, 20).

Regulatory T-cells are another important cell population that were also of interest in this study. Tregs have a range of functions related to the immune response from killing infected host cells, activating other immune cells, and secreting pro-regenerative cytokines (including IL-10), but their role in muscle regeneration in particular is very unique. Tregs promote the migration and proliferation of myogenic cells, activate satellite cells, and promote the phenotypic change from M1 to M2 macrophages and are essential to transitioning into a pro-regenerative state at the wound site (11, 21-23). Satellite cells were also of interest in this study, but were not measured due to time constraints and interruptions by the COVID-19 pandemic. However, their role in pro-regenerative activity is equally as important as IL-10 and T-cells, because they are the local source of myogenic stem cells and will be first cell population to regenerate the muscle fiber population (24). Instead, cells expressing embryonic myosin heavy chain (eMHC), a common marker of myoblast differentiation, were observed to gage IL-10's myogenic ability (25, 26).

In this study, IL-10, in conjunction with reinserted minced muscle (MM), muscle that has been crudely minced and reinserted to the wound site proven to have regenerative benefits (27-29), was injected into Sprague Dawley rats enduring a VML injury to assess its functional recovery and regenerative capabilities, and its observed effects on M1/M2 macrophage polarization, T-cell aggregation, eMHC counts, and fiber size to determine the cellular biology taking place and its efficacy as a possible therapeutic treatment for VML injuries. There is a window in which IL-10 may be effectively delivered to produce optimal results. Early delivery of IL-10 to the wound site 2-4 days following a VML injury exhibits slower regeneration (30). Naturally, too late delivery would be just as ineffective. Delivery of IL-10 must mimic the wound healing response that delays endogenous IL-10 production to accommodate the functions M1 macrophages and their transition to becoming M2 macrophages.

2. Materials and Methods

2.1 VML Injury and IL-10 Delivery

An 8mm biopsy punch was used to create a VML defect 3mm deep into the center of the tibialis anterior (TA) muscle of the left leg (LTA). The missing tissue constituted around 20% of the TA mass. The MM was prepared by hand-mincing the biopsied muscle with scalpels and scissors to be implanted back into the defect site. The tibialis anterior of the right leg (RTA) did not undergo a VML defect to serve as the uninjured control. The proportion of the rats (n=28) received either 2000 ng/ml of IL-10 (n=14) or an equal concentration of PBS injection (n=14) beginning 7 days after injury and received 100µL every other day until day 14, a total of 4 injections.

2.2 Force Data Collection

On the day of tissue harvest, rats were anesthetized using 1.5-2% isoflurane and the hind leg was stabilized at 90° of knee flexion where the tibia was parallel to the bench top. The foot was secured to a force pedal transducer system (Aurora Scientific) with surgical tape, and the peroneal nerve was stimulated with electrodes (150 Hz, 0.1 ms pulse width, 400 ms pulse train) using an S88 physiological simulator (Grass Technologies) to induce the contraction of the TA and produce force on the pedal. The tendon of the extensor digitorum longus muscle was cut to eliminate its contribution during force data collection. Mean Isometric torque average of 5 contractions was expressed as N/kg body weight to determine absolute functional capacity and muscle wet weight (g/kg body weight) was measured to assess functional quality, both numbers were normalized to the control group during data analysis. While still under anesthesia, RTAs and LTAs were harvested, rinsed with sterile PBS, dried, weighed, and frozen and stored at -80°C for later histology analysis.

2.3 Histology Analysis

Frozen tissue samples were thawed to -22°C in a cryotome. Samples were mounted with OCT compound on the stage of the cryotome and sectioned at the site of the defect for LTA samples and sectioned around halfway through the RTA tissue samples since there was no defect to detect. Sections were collected on a slide and the slides were stained with fluorescent markers to be observed under a fluorescent microscope. All slides were stained for the nuclei marker DAPI and ECM protein laminin, but separate cellular markers CD3e, CD68, CD163, and eMHC stained for T-cells, M1 macrophages, M2 macrophages, and myoblasts respectively to be quantified. CD68 stains for both M1 and M2 macrophages, while CD163 is an M2 specific marker. Fiber size was quantified using a custom ImageJ script. All images were taken near the defect site.

2.4 Statistical Analysis

Treatment groups were compared using a two tailed t- test ($P < 0.05$) on the average counts of T-cells, M2 macrophages, eMHC, fiber size, and functional/mass recovery. All graphs represent mean values with standard deviations.

3. Results

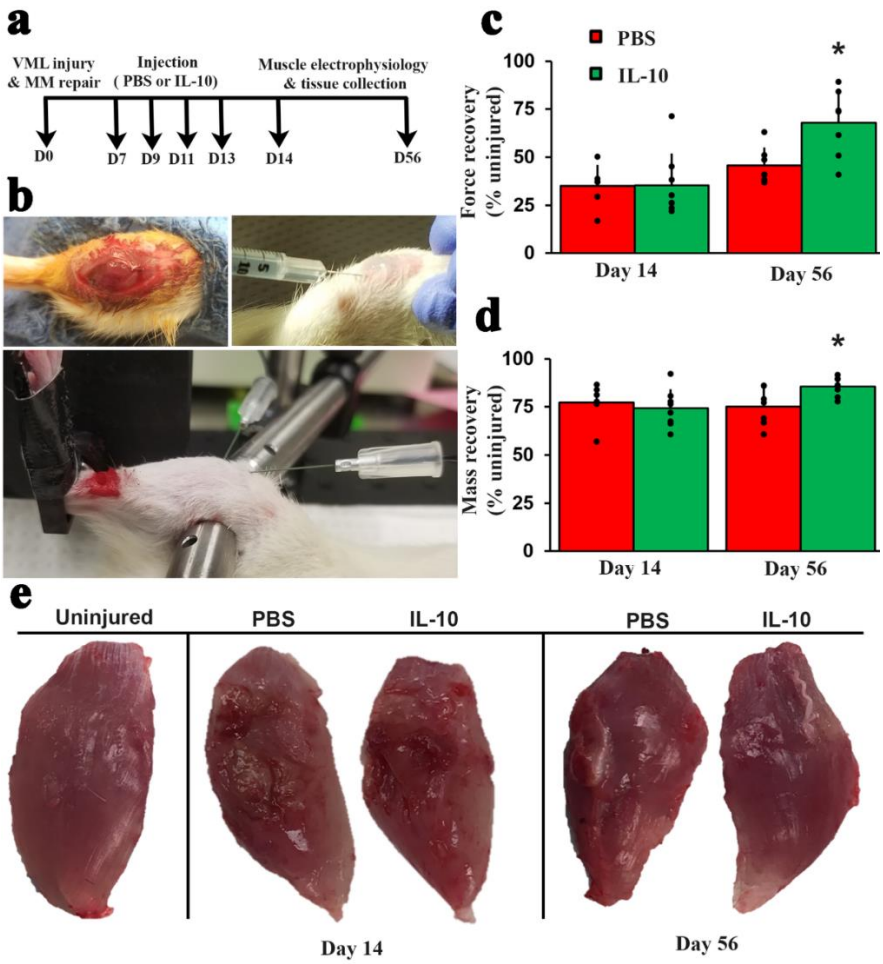

Figure 2: (a) Timeline of VML injury and repair, injection, and force data and tissue collection. **(b)** Images of the MM reinsertion, treatment injection, and TA force data processes**. (c)** The normalized peak contractile torque (N/kg body weight) and **(d)** muscle wet weight (g/kg body weight) of the TA were compared to the uninjured control in both IL-10 treated and PBS treated groups at 14 and 56 days following injury. **(e)** Comparison of PBS and IL-10 treatment groups at days 14 and 56.

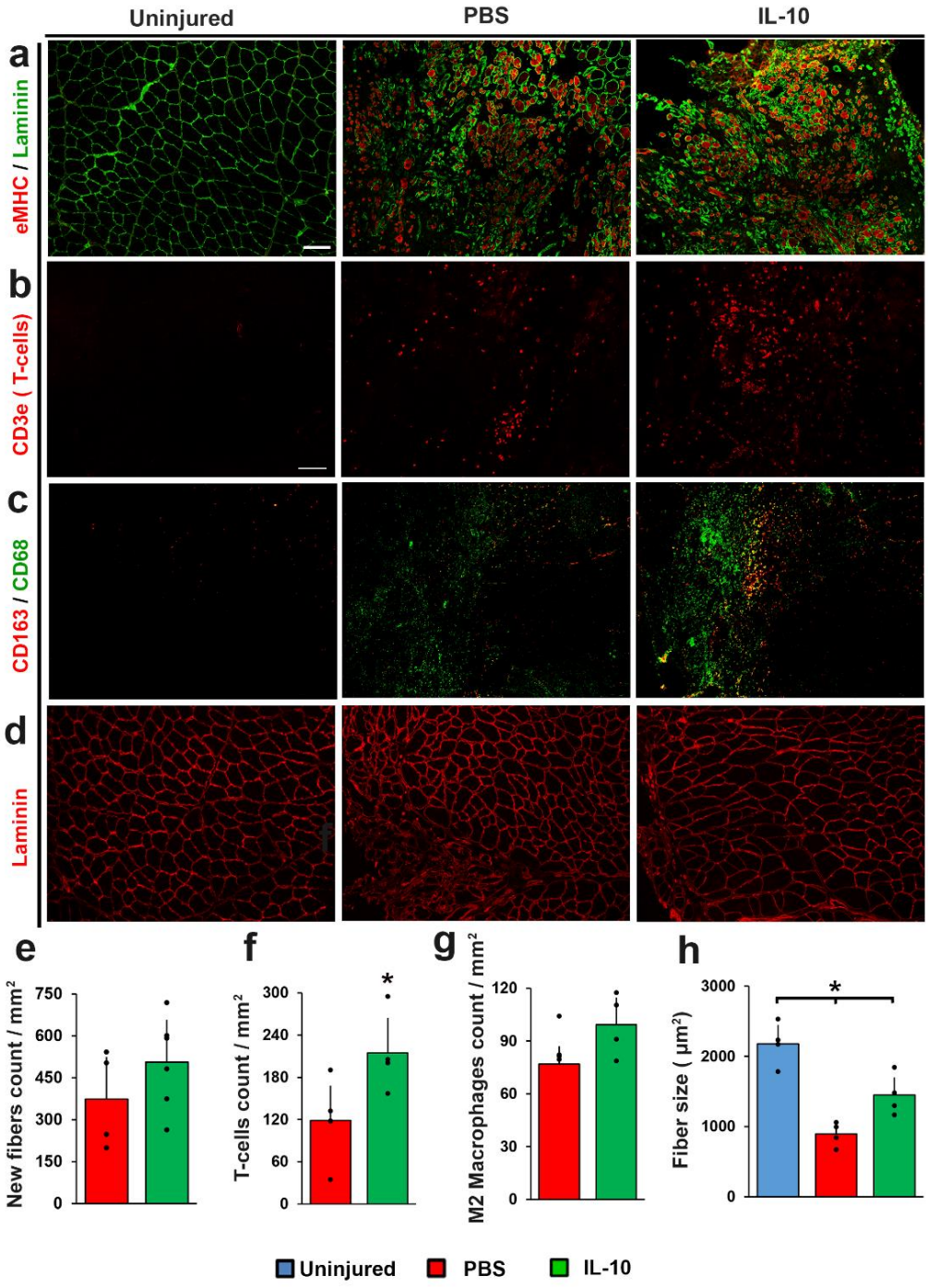

Figure 3: TA muscle cross-sections were stained for markers **(a)** eMHC (red), laminin (green) **(b)** CD3e (red), **(c)** CD68 (green), and CD163 (red) 14 days following injury. **(d)** laminin (red) 56 days following injury. a/d Scale bar = 100µm, b/c scale bar = 250 µm. Tissue sections were counted by hand to quantify **(e)** new fiber formation, **(f)** T-cell aggregation, **(g)** and M2 macrophage polarization. **(h)** muscle fiber cross-sectional area was quantified using a custom ImageJ script. Group means and standard deviations are presented; n=7/group. * denotes statistically significant ($p<0.05$) differences between groups.

The outcomes of this experiment were a significant increase in T-cell aggregation and muscle fiber size, while no observable significant increase in M1/M2 macrophage polarization and new fiber counts (figure 3). Visual accounts comparing the uninjured control and IL-10/PBS treatment groups support these points showing a distinct difference in the number of CD3e stained T-cells present on the histology sections (figure 3b), while in figure 3a and 3c, there is less differences in the number of marked eMHC and M1/M2 macrophages between the experimental and control groups. Significant increases in both functional and mass recovery was also observed in tissue 56 days post injury (figure 2).

4. Discussion

Re-summarizing the results found in figures 2 and 3, significant increases in force/mass recovery after 56 days as well as T-cell and fiber size were observed, while no significant increases in progenitor cell recruitment and differentiation and M2 macrophage counts were observed. The little to no improvement of functional and mass recovery at 14 days was to be expected since the wound site was most likely still within the inflammation stage. The significant improvements at 56 days may most likely be attributed to both the increased fiber sizes, that may constitute higher contractile force measured in the force testing phase, and the increased T-cell infiltration responsible for mediating a pro-regenerative state. With more T-cells present, more pro-regenerative cytokines are secreted, and more pro-regenerative cells are recruited such as new M2 macrophages and myoblasts.

Though not statistically significant, the increases in M2 macrophages and eMHC present in the IL-10 treatment group compared to the PBS and uninjured controls, may also have contributed to increased functional and mass recovery. The rise in both of these factors is most likely influenced by the heightened number of T-cells. It may be possible that the number of M2

macrophages *is* statistically significant, but the window to observe this significant increase may have been more or less than 2 weeks. The same could be said for the number of eMHC counted. There is clearly a comparable difference in the number of new fibers present in the IL-10 group vs the PBS control, perhaps there was another window to observe a statistically significant increase as well. It is known T-cells activate local myogenic satellite cells and influence macrophage phenotype, so it comes somewhat as a surprise that neither of these factors were also statistically significant despite how large the role of T-cells are in muscle regeneration and the near double amount of T-cells present (figure 3f) (21, 22). On the other hand, the wound healing process is a highly complex process, each aspect influenced by several other factors and the interactions between one another. No one alteration to the process should heavily influence other aspects of it, and rightfully so to prevent catastrophe.

Though they were not successfully measured in this study, satellite cells were most likely present in the wound site observed for reasons previously mentioned. It would have painted a better picture of the mechanisms at hand and may have strengthened T-cells roll in mediating the effects of exogenously delivered IL-10 Myoblasts may also be recruited by IL-4 secretion, so a possible solution to further promote an increase in eMHC counts would be to inject a family of pro-regenerative interleukins, including IL-10 and IL-4, in conjunction with MM grafts in order to provide a more holistic regenerative therapy (31).

5. Conclusion

IL-10 and MM show promising results of increases in pro-regenerative cell populations across the board and substantial functional and mass recovery to hopefully become a possible treatment for VML injuries. More must be done to further explore T-cells involvement in this process and ironing out some of the logistics of making it a viable therapeutic treatment, such as

limiting the frequency of injections and finding ways to further promote the treatment's regenerative function, but it is a great stride towards developing a pro-regenerative standard clinical treatment for VML injuries. VML injuries are lifetime debilitation conditions, therefore a regenerative treatment would not only provide an independently functional life for the hundreds of thousands waiting for such treatment but open the doors for applying this technology to various other muscular injuries.

6. Future Directions

Future studies will be focused on further exploring the cell populations at play and their interactions in a VML context. Satellite cells are known to be activated by regulatory T-cells and were previously mentioned as a population of interest for this study. They were attempted to be observed by staining for the cellular marker Pax-7, however those attempts failed, and the development of the COVID-19 pandemic offered little time to follow up with additional attempts. Though IL-10 itself does not provide significant myogenic effects, T-cells activate satellite cells which do have myogenic properties, so studying the cascading effects of IL-10 on satellite cell activation could be valuable. Further studies on the effect of IL-10 and T-cells on the types of myogenic progenitors that migrate to the injury site such as mesenchymal stem cells and pericytes could also aid in the understanding of the VML process. There was no myogenic cell population to compare to the eMHC counts observed, it would be important to know what myogenic cell population from which the myoblasts were being derived. Further exploration on T-cells function would also improve this study as well as attempting to detect the possible windows of statistically significant increases in M2 macrophage and eMHC counts.

7. Acknowledgements

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