Dissecting the Sex-Specific Relationship Between Muscle Damage and Regenerative Myofiber Characteristics in Cardiotoxin-Induced Mouse Models

Naitik Jhanwar[1], Andrea Alfaro-Chacon[2]
1. All Saints Cathedral School, 12th grade, naitikjhanwar@gmail.com
2. University at Buffalo, abalfaro@buffalo.edu

Abstract

Skeletal muscle regeneration following acute injury relies on highly coordinated interactions between inflammatory signaling and muscle tissue responses. Following myofiber necrosis, immune cells, including regulatory T cells, infiltrate the damaged ECM and secrete growth factors like Amphiregulin (Areg), which can promote satellite cell (SC) proliferation and muscle repair. However, how biological sex and Areg genotype are associated to influence these regenerative processes remains poorly defined. We examined whether the relationship between tissue damage and histological measures of regeneration differed according to sex and Areg genotype. It was hypothesized that in a mouse model of CTX-induced inflammatory skeletal muscle injury, female and male mice will differ in the relationship between damaged area and regenerative myofiber characteristics (size and central nuclei), reflecting sex-specific inflammatory and reparative responses. Twenty-seven mice were divided across four experimental cohorts: wild-type (WT) males, WT females, Areg knockout (KO) males, and KO females. Following cardiotoxin (CTX)-induced injury, Tibialis Anterior sections were harvested at day 5, stained with Wheat Germ Agglutinin (WGA) and DAPI, imaged, and analyzed using FIJI to quantify morphometric parameters. Pearson correlation analyses were performed separately for each group. A strong, statistically significant inverse correlation between percent damaged area and median CSA was observed only in WT males ($r = -0.89$, $p = 0.019$). Conversely, the percent damaged area positively correlated with the percent central nuclei in WT females ($r = 0.87$, $p = 0.011$) and KO females ($r = 0.83$, $p = 0.041$). The remaining correlations showed directional trends but did not reach statistical significance ($p > 0.05$). These findings suggest that the relationships among tissue damage, myofiber CSA, and central nucleation do not vary uniformly across biological sex and Areg genotypes. Consequently, relying on a single histological marker is insufficient, highlighting the need for multivariate assessment criteria when evaluating muscle regeneration.

1. Introduction

1.1 The Muscle Regeneration Process

The muscle regeneration process consists of five main phases: muscle degeneration, inflammation, regeneration, maturation, and functional recovery. It begins after injury-induced degeneration, in which damage to the sarcolemma causes cell death, leading to organelle dysfunction, necrosis, and loss of normal muscle fiber structure. In order for the muscle to restore and structurally heal itself back, it must undergo the three following phases: inflammation, regeneration, and maturation. Together, these phases facilitate clearing of damaged muscle fibers, functional recovery, and support formation of neuromuscular junctions.

The inflammation phase is a sterile inflammatory response to tissue damage where immune cells clear cellular debris and signal for danger. This minimizes infection by ensuring that no exogenous agents are involved. Neutrophils are among the first cells to dominate the lesion site, which clear the damaged area through phagocytosis, releasing free radicals and proteases as well as the secretion of cytokines that support inflammation. The cytokines stimulate macrophages, categorized into M1 and M2 macrophages. M1 are pro-inflammatory macrophages that clear more debris whereas M2 are pro-regenerative macrophages that support the formation of new tissue. M1 and M2 are the different macrophage activation stages of phenotypes. In other words, a macrophage in the skeletal muscle regeneration process starts out pro-inflammatory (M1), and changes its profile to pro-regenerative (M2) where it stops secreting cytokines and begins secreting Hepatocyte growth factor and Insulin-like growth factor-1 (Choi et al., 2019; Rigamonti et al., 2014). The shift from M1 to M2 macrophages is very crucial because it facilitates the transition from injury to repair.

The transition from inflammation to regeneration is supported by several signalling molecules, such as Amphiregulin (Areg), a pivotal molecular coordinator to muscle healing (Burzyn et al., 2013). Areg is a ligand that binds to and activates the epidermal growth factor receptor (EGFR). During the resolution of the inflammatory response, Areg is actively secreted into the microenvironment by immune cell populations involved in tissue repair, including accumulating regulatory T (Treg) cells and repair-associated macrophages (Gama et al., 2022). Through paracrine signalling, Areg can act on nearby cells within the regenerating muscle microenvironment, including muscle satellite cells. Rather than simply suppressing inflammation, Areg-mediated EGFR signalling can influence satellite cell activation and proliferation, thereby providing a robust population of progenitor cells. Thus, Areg represents one of the mechanisms through which the changing immune environment can help coordinate the transition from inflammation to muscle generation.

The next restorative phase, regeneration, depends mainly on muscle stem cells, also known as satellite cells (SCs), which remain dormant under normal conditions and activate after injury. Once activated, stem cells can proliferate through either symmetric or asymmetric proliferation. Symmetric division produces two similar unspecialized daughter cells which expand the satellite cell pool, whereas asymmetric division produces one unspecialized daughter cell that maintains the SC population and one specialized daughter cell (a myoblast) destined to differentiate and

fuse into existing or new myofibers. This process aids to the replacement of necrotic fibers with regenerating myofibers characterised by the appearance of centrally located nuclei.

Fibroadipogenic Progenitors (FAPs) also contribute to muscle regeneration and repair but perform a function distinct from that of satellite cells. These cells are located within the spaces between muscle fibers and release signals that aid SCs in working more efficiently. However, their activity must be carefully regulated, because prolonged activation can result in unwanted fat or scar tissue. Therefore, a coordinated balance between satellite cells and FAPs is necessary to successfully regenerate the injured tissue.

Understanding these physiological repair mechanisms is clinically vital because disruption of the normal regeneration cycle contributes to progressive muscle damage in several chronic inflammatory diseases such as polymyositis and dermatomyositis and regenerative muscle disorders such as muscular dystrophy (Deprez et al., 2023). Unlike acute muscle injury where inflammation is normally resolved as regeneration progresses, chronic muscle disorders subject the tissue to persistent inflammatory signaling. This prolonged inflammatory environment can disrupt the transition from pro-inflammatory (M1) activity to pro-regenerative macrophage (M2) activity. The inhibition of the M1 to M2 shift impairs the Areg-mediated signaling necessary to drive stem cell function, thereby interfering with the coordinated regeneration process from tissue injury to repair. At the same time, constant cycles of degeneration and regeneration exhausts the quiescent satellite cell pool while causing dysregulated FAP proliferation. Together, these alterations can prevent effective myofiber regeneration and contribute to the progressive loss of normal muscle structure and function. Therefore, dissecting the exact cellular dynamics that govern muscle injury repair may provide fundamental insights needed to develop remedies that can restore endogenous regeneration in pathological muscle states. It's also important to explore and identify any biological factors, including sex, that may influence these responses.

Maturation refers to the growth and structural organization of the myofibers for their functional development. It can be identified as the stage where centrally located nuclei are shifted to the periphery of the myofibers, just beneath the sarcolemma. Maturation focuses on creation of new myofibers and the successful repair of existing ones. However, for this to occur, the extracellular matrix (ECM), blood vessels, and nerves must also be repaired. The ECM acts as a scaffold that provides structural stability for the formation of new fibers. Within the ECM, collagen is carefully organized and aligned to achieve high muscle contractility and strength. However, excessive collagen accumulation can lead to fibrosis, resulting in muscle stiffness and impaired muscle function. Therefore, controlled ECM remodelling is essential for successful muscle maturation.

1.2 Cardiotoxin (CTX) and Its Effects

To monitor and investigate skeletal muscle regeneration experimentally, researchers require controlled models to induce rapid skeletal muscle injury. Cardiotoxin (CTX) is a commonly used degenerative myotoxin that can cause acute muscle damage. Derived from the venom of *Naja pallida* (the red spitting cobra), CTX is an amphiphilic myotoxin that disrupts skeletal muscle fibers and causes myofiber degeneration. In muscle regeneration research, highly reproducible injury models are essential because variability due to non-uniform injuries and extenuating

factors introduces noise that makes it difficult to determine whether variations reflect experimental inconsistency or true underlying biological differences. Standardizing the initial injury ensures that any differences in outcomes, including sex-specific variations in muscle damage, regenerating myofiber size, and repair progression, are interpreted with greater confidence.

A benefit of using CTX over other forms of injury-inducing methods, such as physical trauma, is that the injury is controlled and sterile. CTX primarily damages myofibers while essential physiological processes for regeneration are preserved. Because the innervation, tendons, vascularization, and satellite cells (SCs) are not damaged, tissue damage remains uniform, standardized, and highly reproducible. Thus, CTX induces substantial muscle fiber degeneration without completely disrupting the structures needed to support the repair.

Following administration, CTX prompts necrosis in the muscle in approximately 1-2 days. It does so by affecting membrane calcium-binding sites in skeletal muscle cells. As an amphiphilic peptide, CTX inserts directly into the lipid bilayer, disrupting the structural integrity of the sarcolemma, forming extremely tiny pores in it. These pores allow an uncontrolled intracellular influx of extracellular calcium ions.

This rapid influx of calcium ions collapses the resting membrane potential (RMP) across the sarcolemma, causing immediate membrane depolarization and uncontrolled, involuntary muscle contractions. The resulting cytosolic calcium overload hyperactivates calcium-dependent proteases, leading to structural breakdown, myofiber lysis, acute muscle injury, and widespread necrosis. Importantly, because the basal lamina and satellite cell microenvironment remain intact despite this initial fiber destruction, this rapid degeneration triggers a highly synchronized, predictable wave of satellite cell activation, directly leading to the systemic phases of inflammation, creation of new myofibers, and muscle regeneration (Wang et al., 2022).

To create a localized injury while limiting systemic exposure, CTX is administered by direct intramuscular injection into the target muscle region. Localizing the myotoxin prevents diffusion into other muscles and cardiac toxicity, allowing the mouse to remain healthy and active while creating a bounded injury. Furthermore, this administration route establishes a framework for studying the regenerative model. Following intramuscular injection, peak myofiber degeneration and acute macrophage infiltration dominate the first 1 to 3 days. By days 5 to 7 post-injury, SC proliferation peaks and early myofiber formation begin, marked by the prominent appearance of centrally located nuclei. Finally, between days 14 and 28, the tissue completes structural maturation, characterized by peripheral nuclear repositioning and restoration of contractile capacity. Establishing this predictable timeline allows researchers to cross-sectionally image the muscle at precise developmental intervals to directly quantify damage and track regenerative metrics. In the context of this study, this approach provides a framework to determine whether the characteristic features of muscle damage progression and regeneration differ between male and female mice (Fu et al., 2023).

Among the skeletal muscles used in CTX-induced injury models, the tibialis anterior is commonly selected because it is accessible for injection and consistent histological analysis (Feno et al., 2023).

1.3 Tibialis Anterior Muscle

The Tibialis anterior (TA) muscle is one of the primary hindlimb muscles used in mouse models of CTX-induced injury and regeneration. Anatomically located on the anterior of the lower hindlimb, the TA muscle is particularly well-suited for experimental manipulation because it is superficial and easily accessible, as shown in Figure 1. This allows CTX to be delivered precisely via controlled and repeatable intramuscular injections directly into the muscle belly (Guardiola et al., 2017).

The TA muscle consists of predominantly fast-twitch glycolytic and slow-twitch oxidative myofibers. This mixed and heterogeneous fiber distribution makes it an ideal representative model for studying skeletal muscle injury and regeneration compared to highly specialized muscles.

Anatomically, the TA is a well-defined muscle unit enclosed in a distinct fascial compartment without internal partitioning. This structural uniformity ensures that when CTX is injected, the solution diffuses evenly throughout the tissue rather than getting trapped in isolated subsections, resulting in an ideally consistent, highly reproducible injury profile (Hardy et al., 2016). The defined boundaries of the TA make sampling and comparison easier.

The TA muscle is also well-suited for histological processing and fluorescence microscopy. Because the muscle fibers run largely parallel to one another in a uniform direction, the muscle can be harvested and oriented consistently for cross-sectional freezing and slicing (cryosectioning). Cross-sections of the TA show distinct, circular myofiber boundaries that are easy to delineate using membrane markers like wheat germ agglutinin (WGA) (Kostrominova, 2011). Additionally, its compact, fusiform geometry allows researchers to capture high-resolution whole-muscle cross-sections in single or tiled images. This enables comprehensive, automated quantification of damaged areas and centrally located nuclei across the entire muscle tissue.

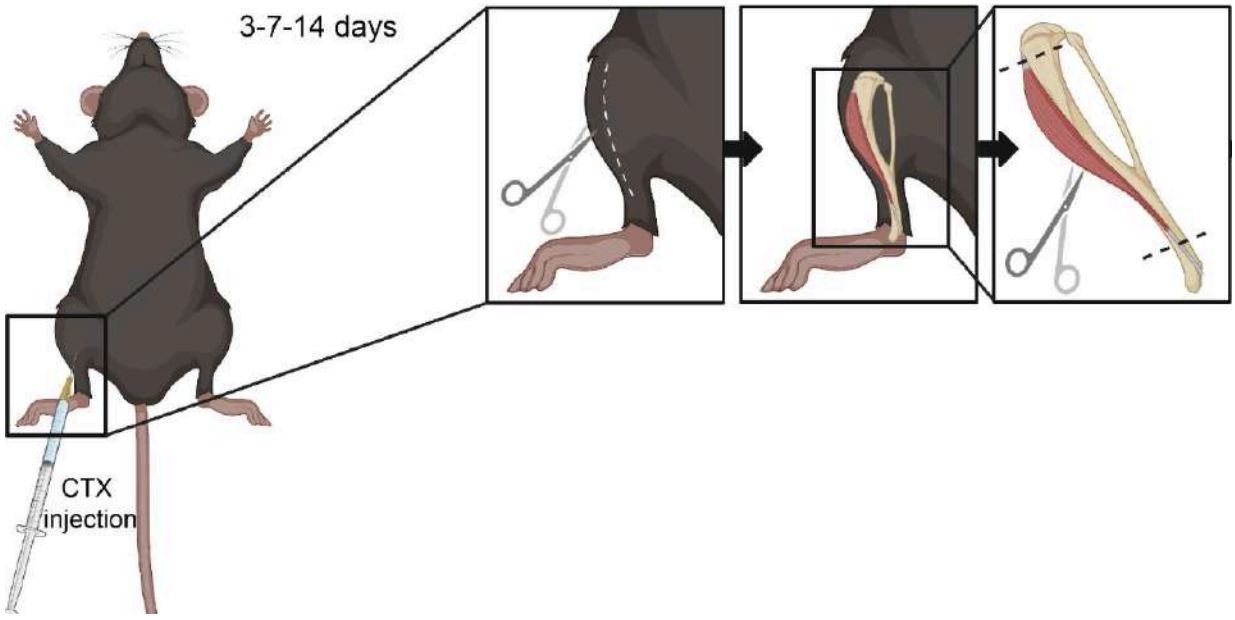

Figure 1. CTX injection in the TA muscle.

1.4 Wheat Germ Agglutinin/DAPI Staining

WGA is a wheat grain protein that binds specifically to N-acetylglucosamine and sialic acid residues, which are abundant in the extracellular matrix and plasma membranes of mammalian cells. When conjugated to a fluorescent marker, WGA can be used to outline individual myofiber boundaries. DAPI is a fluorescent stain that binds strongly and specifically to adenine-thymine (A-T) rich regions in DNA, allowing nuclei to be visualized within the muscle section (Kapuscinski, 1995). Together, these two stains provide structural and nuclear information from the same muscle cross-section.

The stained muscle cross-sections were imaged using fluorescence microscopy. In the images, DAPI was visualized in blue, and fluorescently labeled WGA was visualized in green.

By selectively targeting carbohydrate chains on the sarcolemma, fluorescently labeled WGA outlines the physical borders of individual muscle fibers in green. This outline provides essential spatial definition needed for image analysis software to map muscle cross-sections, measure fiber cross-sectional area, and calculate the percent of damaged myofiber area by identifying areas where membrane integrity has been disrupted or replaced by extracellular matrix breakdown (Kostrominova, 2011).

DAPI shows the spatial distribution of all myonuclei within the muscle tissue. In mature, undamaged skeletal muscle fibers, myonuclei are normally positioned at the outer periphery beneath the sarcolemma. Following cardiotoxin-induced injury, active muscle regeneration causes newly forming myofibers to display centrally located nuclei. By pairing DAPI with WGA boundary staining, researchers can accurately identify and count these central nuclei relative to total myofibers, establishing a commonly used histological indicator of muscle regeneration as shown in Figure 2.

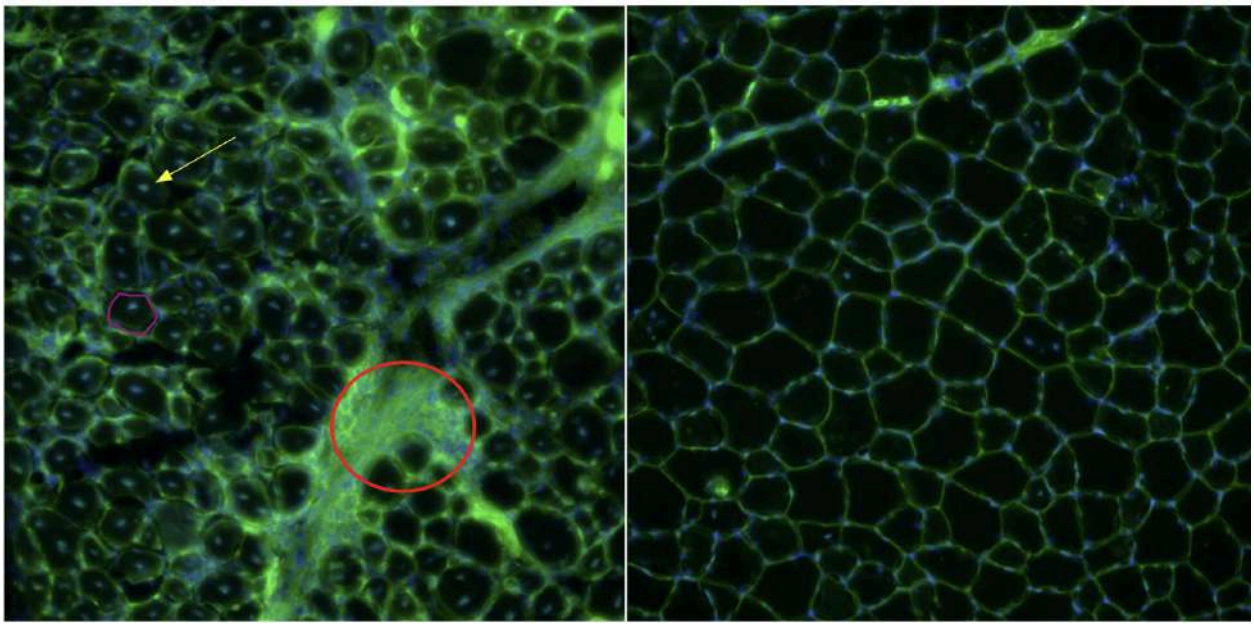

Figure 2a (left) and Figure 2b (right) depict cross-sectioned muscle from an injured and uninjured mouse, respectively. Common measurements of muscle repair are the damaged area (red circle), central nuclei (yellow arrow), and size of myofiber (magenta polygon) in Figure 2a. In the non-damaged muscle in Figure 2b, there is no damaged area, the myofibers' area are homogenous, and the myofibers with central nuclei are minimal.

1.5 Research Gap and Research Question

Despite significant advances in understanding the cellular mechanisms of skeletal muscle repair, a critical gap remains in how biological sex modulates the relationship between acute injury severity and regenerative capacity. Most preclinical muscle injury studies have historically relied predominantly or exclusively on male animal models, assuming that regenerative pathways operate identically across sexes.

Consequently, potential sex-specific differences in immune cell infiltration, satellite cell dynamics, and growth factor responses remain poorly characterized. This lack of female representation creates a fundamental problem in biomedical research, as sex-specific responses may lead to variable outcomes in human muscle trauma and disease. Therefore, to address this gap, this study aims to systematically compare male and female responses in a standardized injury model.

In the study, muscle regeneration is investigated using histological characteristics such as myofiber cross-sectional area (CSA) and central nuclei along with the extent of muscle damage. The Areg genotype is also represented in the experimental cohort, so it needs to be considered when interpreting the sex-associated differences.

Specifically, this paper seeks to answer the following research question: In a mouse model of Cardiotoxin (CTX)-induced inflammatory skeletal muscle injury, how does sex influence the correlation between the extent of tissue injury and indices of regeneration?

2. Methodology

2.1 Animals and Experimental Groups

All experimental procedures were conducted using young adult C57BL/6 mice aged between 8 and 10 weeks. To evaluate the roles of biological sex and Amphiregulin (Areg) expression in acute muscle repair, the study cohort comprised both wild-type (WT) and Areg knockout (KO) animals across both sexes. The experimental groups consisted of 6 WT male mice, 7 WT female mice, 8 KO male mice, and 6 KO female mice, giving a total of 27 animals. Standardizing the age range between 8 and 10 weeks ensured that all mice were within a consistent post-developmental adult stage with fully mature, responsive satellite cell populations at the time of injury induction.

Eight- to ten-week-old male and female C57BL/6 mice were obtained from Taconic Farms (Germantown, NY). All procedures involving mice were reviewed and approved by the Institutional Animal Care and Use Committee at the University at Buffalo.

2.2 CTX-Induced Tibialis Anterior Muscle Injury

To establish a standardized model of acute skeletal muscle injury, a 30 μg/mL dose of cardiotoxin (CTX) was administered via direct intramuscular injection into the belly of the TA muscle. By standardizing this exact injection volume across all 27 experimental mice, an equal baseline of initial tissue damage was established regardless of sex or Areg genotype.

2.3 Tissue Collection and Cryosectioning

Five days after CTX injection, the TA muscles were harvested and immediately snap-frozen. The frozen tissue was then cryosectioned at a thickness of 20 μm for subsequent staining and fluorescent imaging.

Day 5 post-injury was selected to assess early muscle regeneration, including the appearance of centrally nucleated myofibers (Pizza & Buckley, 2023).

2.4 WGA and DAPI Staining

Following tissue cryosectioning, the TA muscle sections underwent dual-fluorescent staining using wheat germ agglutinin (WGA) and DAPI to visualize and delineate myofiber boundaries and cellular nuclei, respectively.

Sections were incubated with WGA at a dilution of 1:500 for 30 minutes at 37°C. This provides the necessary heat and time for optimal binding of the lectin to N-acetylglucosamine and sialic acid residues along the sarcolemma and extracellular matrix. Sections were subsequently stained with DAPI at a dilution of 1:1000 for 2 minutes at room temperature, to visualize myonuclei without over-saturating the fluorescent signal.

2.5 Fluorescence Imaging

To capture high-resolution cross-sectional images of the stained tissue, slides were imaged using a Leica DMi8 fluorescence microscope equipped with a 20x magnification. For each TA muscle section, non-overlapping fields of view were captured using filter sets appropriate for the fluorescent WGA and DAPI signals. WGA fluorescence was used to delineate individual myofiber boundaries while DAPI fluorescence was used to identify nuclei.

Imaging at 20x magnification provided an optimal balance between field of view and spatial resolution, capturing a sufficient number of individual myofibers per image to assess tissue structure while maintaining the high optical clarity required to accurately identify centrally located nuclei and measure individual myofiber cross-sectional areas.

2.6 FIJI Image Analysis

Digital image analysis was performed using FIJI (an expanded distribution of ImageJ software) to quantify muscle damage and regenerative outcomes from the fluorescence images. Using the dual-channel fluorescent images, specific metrics were extracted to evaluate tissue architecture and repair progress for each image.

First, the total count of myofibers exhibiting centrally located nuclei was determined to calculate the proportion of actively regenerating fibers within each field of view. The number and percentage of centrally nucleated myofibers were quantified as an indicator of muscle regeneration.

Next, the percentage of damaged muscle area was quantified relative to the total cross-sectional muscle area by measuring regions where sarcolemmal WGA staining revealed membrane disruption, necrotic debris, or expanding extracellular matrix compared to intact tissue.

Finally, individual myofiber boundaries defined by WGA fluorescence were traced to measure the cross-sectional area (CSA) of each fiber in square micrometers (μm^2). The number of central nuclei per myofiber was also recorded for the correlation analysis.

2.7 Statistical and Correlation Analysis

Correlation analyses were performed to examine the relationships between tissue injury severity, myofiber morphology, and active regeneration. Four specific correlation analyses were performed:

1. Percent damaged area versus myofiber cross-sectional area (CSA): Evaluated whether the severity of localized tissue destruction was correlated with the size (growth or atrophy) of surviving or newly forming myofibers.
2. Central nuclei per myofiber versus myofiber CSA: Checked the relationship between the absolute number of myonuclei recruited to the center of a fiber and that fiber's physical size, indicating whether larger regenerating fibers contain a higher density of central myonuclei.

3. Percent central nuclei versus myofiber CSA: Saw whether the overall proportion of regenerating fibers in a given field of view correlates with average fiber growth, linking the regional density of regeneration to overall fiber hypertrophy.
4. Percent damaged area versus percent central nuclei: Compared the spatial extent of initial muscle damage to the regional rate of active regeneration, determining whether areas experiencing higher damage exhibit a proportional increase in central nucleation.

These correlation analyses were used to identify associations between muscle injury, myofiber morphology, and regeneration. Because correlation does not establish causation, the results were interpreted as relationships between variables rather than evidence that one variable directly caused changes in another. Correlations were evaluated separately across the experimental groups to determine whether the relationships differed between male and female mice and between WT and Areg KO animals.

3. Results

3.1 Animal Baseline and Descriptive Morphometrics

To characterise the overall response to CTX- induced injury, we first examined the experimental cohort and the main morphological features of muscle regeneration post-injury.

3.1.1 Experimental Cohort and Morphological Assessment

A total of 27 mice (8-10 week old) were included across 4 experimental groups: 6 WT males, 7 WT females, 8 Areg KO males, and 6 Areg KO females. To evaluate the correlation of different parameters of muscle regeneration in a CTX model, we ensured that all mice were adults with fully mature, responsive satellite cell populations at the time of injury induction. Direct intramuscular delivery was chosen to confine the myotoxin strictly to the TA muscle tissue, preventing systemic diffusion and avoiding systemic or cardiac toxicity. This localized approach allowed the mice to remain healthy, mobile, and physiologically stable throughout the study.

An optimal concentration of the toxin was administered to induce widespread, uniform myofiber necrosis throughout the single-compartment TA muscle belly without causing excessive fluid pressure that could mechanically disrupt the intramuscular structures. Controlling the extent and severity of the initial lesion in this manner ensured that any variations quantified during image analysis, such as differences in myofiber cross-sectional area or central nuclei, can be confidently attributed to true biological differences in sex-specific regenerative response rather than artifacts of inconsistent injury delivery.

3.1.2 Muscle Damage and Regeneration Parameters

To establish baseline parameters of injury severity and regenerative response, we quantified the percentage of damaged tissue area, the proportion of muscle fibers containing central nuclei, and the median myofiber cross-sectional area (CSA) across wild-type (WT) and Areg knockout (KO) male and female cohorts (Figure 3).

- **Percentage of Damaged Tissue Area (Figure 1a):** WT females exhibited the highest average percentage of tissue damage (~25%), followed by WT males (~18%) and KO females (~20%). KO males displayed the lowest overall damaged area (~11%).
- **Percentage of Muscle Fibers with Central Nuclei (Figure 1b):** Central nucleation rates remained relatively consistent across all four experimental groups, with cohort averages hovering between ~28% and ~33%.
- **Median Myofiber Cross-Sectional Area (Figure 1c):** KO males demonstrated the largest median myofiber CSA (~1700 μm^2). The remaining three cohorts, WT males, WT females, and KO females, exhibited lower and comparable median fiber sizes, averaging around ~1400 μm^2.

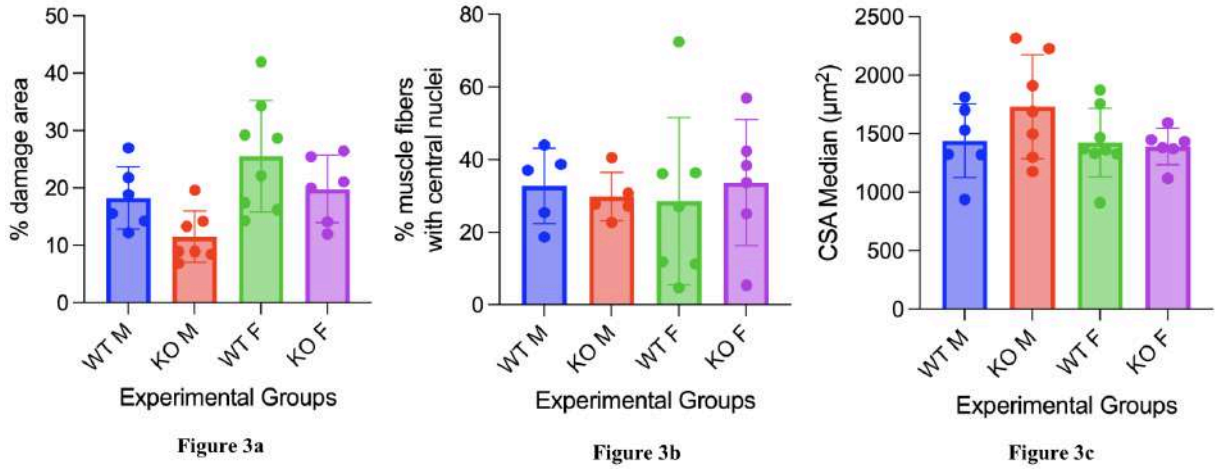

Figure 3. Baseline morphometric parameters in cardiotoxin-injured TA muscle. Quantification of (a) percent damaged tissue area, (b) percent muscle fibers containing central nuclei, and (c) median myofiber cross-sectional area (in square micrometers) across WT male (blue), KO male (red), WT female (green), and KO female (purple) cohorts. Each dot represents an individual biological replicate.

3.2 Primary Correlation Analyses: Injury Severity vs. Regeneration

Correlation analyses were used to assess the extent of CTX-induced damage with regenerative changes in the TA muscle.

3.2.1 Percent Damaged Area vs. Myofiber CSA

To determine whether muscle injury was associated with impaired muscle fiber growth, we evaluated the relationship between the percentage of damaged muscle area and median myofiber cross-sectional area (CSA) (Figure 4). A strong, statistically significant inverse correlation was observed in WT males ($r = –0.89$, $p = 0.019$), indicating that higher muscle damage was associated with smaller muscle myofibers. A moderate negative association was observed in KO females ($r = –0.71$, $p = 0.117$), while WT females demonstrated a weaker inverse relationship ($r = –0.47$, $p = 0.285$). In contrast, KO males displayed a positive regression trend ($r = 0.39$, $p = 0.345$). Although the direction and correlation strength varied across the groups, only the WT male cohort reached statistical significance ($p < 0.05$), indicating that when the percentage damaged is higher, the area of each myofiber is decreased. The data indicates a strong inverse relationship between tissue damage and myofiber size in WT males, whereas a similar relationship is not statistically supported across other groups with the current sample size.

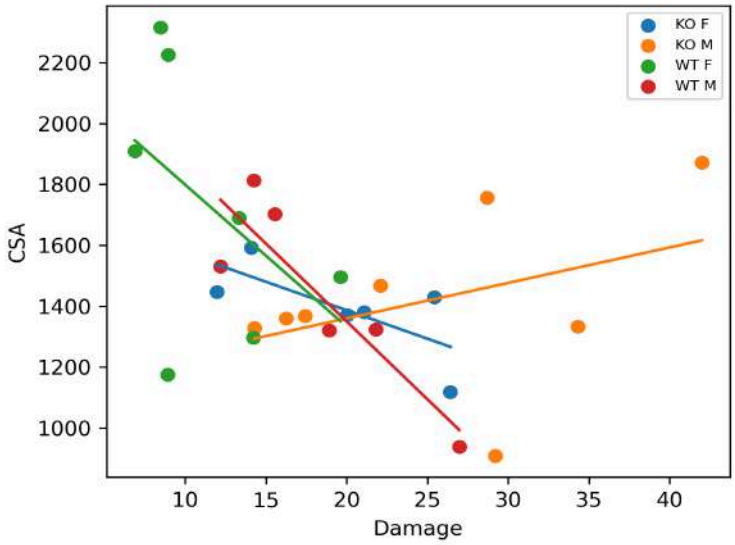

Figure 4. Scatter plots showing the association between the percentage of damaged muscle area and median CSA in WT and Areg KO mice separated by sex. Each point represents one biological replicate. Solid lines indicate linear regression. Pearson correlation coefficients (r) and coefficients of determination (r^2) were calculated independently for each group.

3.2.2 Central Nuclei per Myofiber vs. Myofiber CSA

To determine whether muscle regeneration was associated with myofiber size, we examined the relationship between the number of centrally nucleated fibers per myofiber and median CSA. Negative correlations were observed across all experimental groups (Figure 5), with the strongest trend seen in WT females ($r = –0.65$, $p = 0.112$) and WT males ($r = –0.61$, $p = 0.272$). Similar negative associations were observed in KO females ($r = –0.57$, $p = 0.238$) and KO males ($r = –0.53$, $p = 0.354$). Although the groups showed a similar overall trend of association, none

of the correlations had a significant p-value ($p > 0.05$). Therefore, the data suggests a higher central nucleation tendency occurring associated with lower myofiber CSA, but this relationship is not statistically supported with the present sample size.

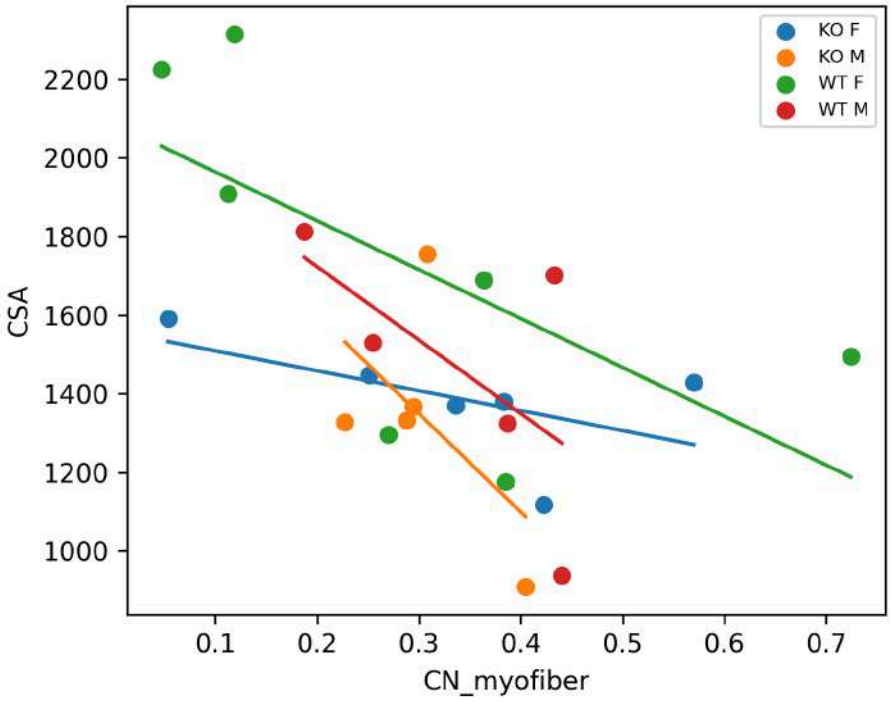

Figure 5. Scatter plots illustrating the association between the number of centrally nucleated (CN) fibers per myofiber and median CSA. Each point represents an individual mouse. Linear regression lines are shown for each experimental group.

3.2.3 Percent Central Nuclei vs. Myofiber CSA

We next evaluated whether the percentage of muscle fibers containing central nuclei was associated with fiber size (Figure 6). Moderate to strong inverse correlations were observed across all groups, with WT males displaying the strongest relationship ($r = –0.77$, $p = 0.126$), followed closely by WT females ($r = –0.64$, $p = 0.126$), KO females ($r = –0.57$, $p = 0.238$), and KO males ($r = –0.52$, $p = 0.368$). Although every group exhibited a consistent negative trend, suggesting that muscles undergoing more extensive active regeneration tend to contain smaller myofibers. None of the correlations reached statistical significance ($p > 0.05$). Consequently, while the directional trend is uniform across genotypes and sexes, it was not statistically supported within the present sample size.

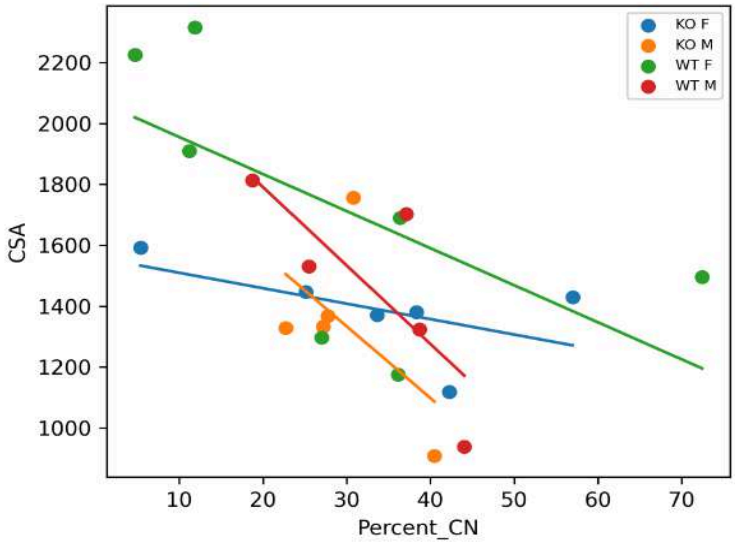

Figure 6. Scatter plots showing the association between the percentage of fibers containing central nuclei (CN) and median CSA in WT and KO mice. Linear regression analyses were performed independently for each sex and genotype.

3.2.4 Percent Damaged Area vs. Percent Central Nuclei

To determine whether localized muscle injury promotes active regeneration, we examined the relationship between the percentage of damaged muscle area and the percentage of centrally nucleated fibers (Figure 7). Strong positive correlations were observed in WT females ($r = 0.87$, $p = 0.011$), KO females ($r = 0.83$, $P = 0.041$), and WT males ($R = 0.82$, $P = 0.088$). KO males also exhibited a moderate positive correlation ($R = 0.51$, $P = 0.379$). Because the regressions for both female cohorts (WT and KO) reached statistical significance ($P < 0.05$), the data indicates that greater tissue damage was significantly associated with heightened regenerative activity. In contrast, while positive directional trends were present in male cohorts, these relationships did not achieve statistical significance, suggesting that the tight alignment between injury severity and central nucleation may be more pronounced or reliably maintained in females. The observed associations differed between Areg WT and Areg KO mice. Additional mechanistic studies are needed to determine whether these differences are directly mediated by Areg signaling. Table 1 is a comparative summary of sex-specific and genotype-specific trends across all of the correlation analyses.

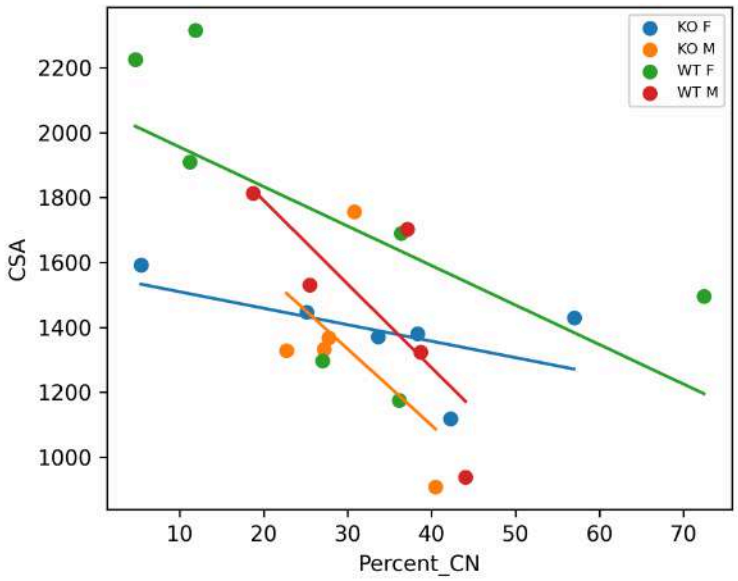

Figure 7. Scatter plots showing the association between the percentage of damaged muscle area and the percentage of fibers containing central nuclei (CN). Linear regression lines were generated for each genotype and sex.

Relationship	Group	n	R	R^2	P value
Damage vs. CSA	WT M	6	-0.886	0.786	0.0186
	KO M	8	0.386	0.149	0.345
	WT F	7	-0.472	0.222	0.285
	KO F	6	-0.706	0.498	0.117
Central nuclei/myofiber vs. CSA	WT M	5	-0.613	0.375	0.272
	KO M	5	-0.534	0.286	0.354
	WT F	7	-0.652	0.426	0.112
	KO F	6	-0.569	0.324	0.238
% Central nuclei vs. CSA	WT M	5	-0.773	0.597	0.126
	KO M	5	-0.521	0.271	0.368
	WT F	7	-0.635	0.403	0.126
	KO F	6	-0.569	0.324	0.238
Damage vs. % Central nuclei	WT M	5	0.822	0.675	0.088
	KO M	5	0.51	0.261	0.379
	WT F	7	0.869	0.755	0.0111
	KO F	6	0.831	0.69	0.0405

Table 1. A summary of the statistical metrics of all four correlation analyses. (From left to right) n values are representative of the number of mice in each genotype. r and r^2 values representing the accuracy of each regression are in subsequent columns. p-values to see for statistical significance are in the last column.

4. Discussion

4.1 Interpretation of Injury and Regeneration Correlations

The significant inverse coupling observed between tissue damage and median myofiber cross-sectional area (CSA) in wild-type (WT) males indicates that myonecrotic injury by CTX suppresses fiber growth in unmanipulated male muscle. In this cohort, larger damaged areas strongly correlated with a predominant population of smaller, newly forming myofibers. However, this relationship was weaker in WT females, moderate in Areg knockout (KO) females, and absent (with a positive trend) in KO males. The failure of Areg KO males to maintain this inverse correlation suggests that Amphiregulin deficiency alters the typical correlation between initial tissue destruction and muscle remodeling.

In contrast to damage vs. size dynamics, the relationships comparing central nucleation against myofiber CSA yielded a highly consistent pattern across all four experimental groups. Whether examining the percentage of centrally nucleated fibers or the absolute number of central nuclei per myofiber, every cohort exhibited negative correlations. This uniform inverse relationship is biologically expected, since acute muscle injury activates resident satellite cells to form myofibers that temporarily retain centrally positioned nuclei before completing hypertrophic maturation. Importantly, because none of these central nucleation vs. CSA correlations reached statistical significance, the data indicates that while central nucleation reliably correlates with smaller fiber dimensions during active repair, this alone doesn't statistically define fine variations in fiber growth across genotypes or sexes at this sample size.

Examining the percent damaged area against the percentage of centrally nucleated fibers revealed a strong positive correlation that was upheld primarily by female cohorts. Both WT and Areg KO females demonstrated significant positive associations between damaged area and the percentage of centrally nucleated fibers. These findings suggest that greater tissue damage was associated with a greater proportion of centrally nucleated fibers at day 5 following CTX injury. Although male cohorts also displayed positive directional trends, their correlations did not achieve statistical significance. Together, these combined findings show that while central nucleation markers reflect standard regenerative biology, the coordination between initial injury severity, active regeneration, and myofiber growth is more specifically related to biological sex and Areg signaling.

The relatively small sample size limits statistical power and may have contributed to the lack of statistical significance in several associations. Future studies using larger cohorts will be needed to determine whether these patterns are reproducible.

4.2 Comparisons With Existing Literature

Baseline regenerative capacity and myofiber size dynamics are highly impacted by biological sex. Research by (Deasy et al., 2007) demonstrated clear sexual differences in satellite cell function, stress responses, and regenerative potential following experimental injury. Furthermore, investigations into muscle fiber heterogeneity confirm that mixed-fiber muscles, such as the Tibialis Anterior, display distinct metabolic and morphological responses to injury

depending on sex-specific hormonal microenvironments, emphasizing the necessity of evaluating male and female cohorts independently in regenerative models (Bahri et al., 2019). These findings support the importance of considering male and female muscle separately when studying regeneration, as differences in the cellular response to injury may affect the repair progression. This is relevant to the present study, where the relationships between tissue damage, central nucleation, and myofiber CSA were not identical across male and female groups.

The inverse relationship observed between tissue damage and myofiber size is also consistent with the established progression of CTX-induced muscle regeneration. (Hardy et al., 2016) and (Wang et al., 2022) demonstrated that acute cardiotoxin (CTX) injury triggers a phase of myofiber necrosis followed by myotube formation, resulting in smaller average fiber sizes during early-to-mid repair. Our WT male data supports this model, with wild-type (WT) males in the present study showing a statistically significant inverse correlation ($r = –0.89$, $p = 0.019$) between percent damaged area and median myofiber cross-sectional area (CSA), indicating that higher damage leaves a higher proportion of small fibers at Day 5 post-injury. KO females showed a similar negative trend ($r = –0.71$, $p = 0.117$) as well, although this relationship was not significant. In contrast, Areg knockout (KO) males exhibited a positive but non-significant trend ($r = 0.39$, $p = 0.345$), where higher damage did not correlate with smaller fiber dimensions. Therefore, the expected negative relationship between damage and fiber size was not maintained uniformly across all groups, suggesting that Areg loss may influence the progression of post-injury muscle remodeling. However, because the KO male correlation was not statistically significant and correlations were not directly compared between groups, this finding should be interpreted as a trend rather than evidence of a male-specific Areg effect.

The differences observed between WT and Areg KO groups can also be considered in relation to previous work on immune-mediated muscle repair. Our findings are consistent with a potential role for Areg in muscle repair; however, the present study does not directly establish an Areg-dependent mechanism. It builds on the work of Burzyn et al., 2013, who showed that Treg-derived Amphiregulin (Areg) increases muscle satellite cell proliferation and muscle repair post-injury. While their study indicated an overall decline in regenerative output upon loss of Areg signaling, our data indicates that Areg KO does not uniformly suppress regeneration across all metrics. Central nucleation remained relatively similar across WT and KO cohorts, while the relationship between damage and CSA varied between groups. KO females, for example, maintained a negative damage-CSA trend, whereas KO males did not. This suggests that the effect of Areg loss may depend on the broader biological environment, including sex, rather than producing the same regenerative response in every group. However, the present study did not directly measure any downstream pathways (e.g. Treg activity, EGFR signaling etc), so the mechanisms responsible for these differences remain to be determined.

Our findings support growing clinical arguments for sex-specific treatments in muscle trauma. Historically, preclinical and clinical knowledge for muscle loss or severe strain injuries have had identical treatment protocols for both male and female subjects. However, existing literature on sex-specific treatment responses, combined with our observation that female muscle maintains tight damage-to-nucleation coupling while male muscle relies heavily on Areg signaling to couple damage with fiber growth, indicates that male and female muscle recover through

different pathways. Therefore, effective regenerative therapies may require different strategies: female patients may benefit more from early anti-inflammatory interventions that limit initial tissue damage, and male patients may require targeted growth factor supplementation (such as Areg or EGFR ligands) to drive satellite cell expansion and myofiber hypertrophy regardless of initial injury size. Further studies using larger cohorts, multiple stages of regeneration, and direct measurements of Areg-EGFR and immune cell signaling are required to determine whether these differences can eventually translate into sex-specific regeneration therapies.

4.3 Real-World Implications

The findings of this study carry potential implications for the field of regenerative medicine, particularly in the development of precision strategies for the treatment of acute muscle injuries. Current clinical protocols for treating severe skeletal muscle trauma, strain injuries, and muscle mass loss predominantly utilize standardized routines that treat male and female patients identically. The different correlation patterns observed between male and female mice suggest that the relationship between tissue damage, myofiber size, and central nucleation may not be identical across sexes. These findings support the need to consider sex as a biological variable when studying muscle repair instead of assuming that regenerative response would be identical across males and females. Optimizing regenerative therapies by taking sex into consideration could improve clinical outcomes. For instance, females could have different therapies than males, and the two different sexes could require different times of intervention. This would also require further studies investigating different stages of regeneration to understand whether the differences could eventually help in designing sex-specific therapeutics.

These results also highlight Areg as an important pathway in the regulation of functional muscle recovery. By demonstrating that the absence of Areg alters the predictable relationship between localized damage severity and myofiber damage, this work reinforces the potential of the Amphiregulin pathway. As Areg can signal through the epidermal growth factor receptor (EGFR) signaling and has been linked to satellite cell function, these findings provide further reason to investigate this pathway in the context of muscle repair and treatment. Such targeted approaches could significantly accelerate tissue repair in patients suffering from severe musculoskeletal injuries or post-surgical tissue deficits. However, the present study did not directly measure EGFR activity or downstream signaling, so additional studies would be required before considering Areg as a therapeutic target.

Furthermore, these insights offer valuable framework considerations for investigating similar mechanisms in degenerative myopathies and age-related muscle wasting. Diseases such as Duchenne muscular dystrophy (DMD) and sarcopenia are characterized by persistent cycles of degeneration that eventually exhaust the endogenous regenerative capacity of satellite cells. The observed decoupling of injury severity from fiber growth in Areg-knockout models mirrors the dysregulated immune-muscle crosstalk seen in degenerative microenvironments. Further investigation of Areg and regulatory T cell signaling can help understand how immune-muscle interactions influence muscle repair during pathological conditions. Insights gained from manipulating Areg could guide the development of immunotherapies targeted to restoring regulatory T cell-mediated signaling, preserving muscle mass, and mitigating progressive atrophy in disease states.

Finally, this study emphasizes the value of adopting multi-variate assessment criteria in investigating muscle regeneration. Traditional evaluations of muscle repair usually rely on isolated metrics, such as central nucleation percentages, to determine the extent of tissue damage that further might have implications on designing the therapeutic strategies. However, the data reveals that baseline central nucleation rates remained mostly uniform across all experimental cohorts despite conspicuous differences in median fiber CSA and spatial damage correlations. This shows that all the measurements do not necessarily change in parallel. Relying just on one single marker could lead to inaccurate conclusions. Therefore, combining correlated profiling, such as combining damage mapping, fiber characteristics, and nuclei positioning, provides a much more reliable standard for evaluating regenerative mechanisms.

5. Future Directions

To build upon the sex- and genotype-specific trends identified in this study, future investigations should incorporate multiple timeframes in sampling after CTX injury. While a single post-injury endpoint provides a snapshot of active repair, analysis at different days such as day 3, 7, 14, and 28 post-CTX injection would clarify whether the uncoupled damage-to-growth relationship in Areg knockout (KO) males reflects a permanent deficit, an accelerated hypertrophic phase, or a delayed maturation timeline. Tracking the progression of central nuclei migration back to the myofiber periphery across these distinct time points would establish whether *Areg* loss fundamentally slows the rate of structural maturation or simply changes fiber dimensions during intermediate repair phases.

Future studies could also expand the experimental workflow by integrating single-cell RNA sequencing to dissect the precise cellular mechanisms driving Areg-associated immune muscle cross-talk. Profiling of resident satellite cells, regulatory T cells (Tregs), and fibro-adipogenic progenitors (FAPs) at peak regeneration intervals would reveal downstream chronological changes in the epidermal growth factor receptor (EGFR) signaling cascade. Comparing chronological profiles between male and female Areg KO cohorts could further identify sex-specific pathways that may protect female muscle from the growth decoupling observed in KO males.

Given that the epidermal growth factor receptor (EGFR) binds multiple ligands beyond Amphiregulin, such as epidermal growth factor, transforming growth factor-alpha, and heparin-binding EGF-like growth factor, the limited differences observed between Areg WT and KO cohorts may reflect functional compensation. Future research should investigate whether expression of alternative EGFR ligands is more prevalent in Areg-deficient muscle tissue following CTX injury.

In conclusion, this work highlights the necessity of incorporating biological sex as a fundamental variable when evaluating skeletal muscle regeneration and growth factor signaling. Our findings demonstrate that male and female muscles exhibit distinct damage vs. growth correlation profiles and distinct sensitivities to Areg loss, which emphasizes that muscle repair mechanisms operate through sex-specific regulatory networks. Recognizing and accounting for

these underlying sex-specific differences will be essential for developing targeted therapeutic strategies and improving preclinical models of musculoskeletal recovery.

References

Bahri, O. A., Naldaiz-Gastesi, N., Kennedy, D. C., Wheatley, A. M., Izeta, A., & McCullagh, K. J. A. (2019). The panniculus carnosus muscle: A novel model of striated muscle regeneration that exhibits sex differences in the mdx mouse. *Scientific Reports*, *9*(1), 15964.

Burzyn, D., Kuswanto, W., Kolodin, D., Shadrach, J. L., Cerletti, M., Jang, Y., Sefik, E., Tan, T. G., Wagers, A. J., Benoist, C., & Mathis, D. (2013). A special population of regulatory T cells potentiates muscle repair. *Cell*, *155*(6), 1282–1295.

Choi, W., Lee, J., Lee, J., Lee, S. H., & Kim, S. (2019). Hepatocyte Growth Factor Regulates Macrophage Transition to the M2 Phenotype and Promotes Murine Skeletal Muscle Regeneration. *Frontiers in Physiology*, *10*, 914.

Deasy, B. M., Lu, A., Tebbets, J. C., Feduska, J. M., Schugar, R. C., Pollett, J. B., Sun, B., Urish, K. L., Gharaibeh, B. M., Cao, B., Rubin, R. T., & Huard, J. (2007). A role for cell sex in stem cell-mediated skeletal muscle regeneration: female cells have higher muscle regeneration efficiency. *The Journal of Cell Biology*, *177*(1), 73–86.

Deprez, A., Orfi, Z., Rieger, L., & Dumont, N. A. (2023). Impaired muscle stem cell function and abnormal myogenesis in acquired myopathies. *Bioscience Reports*, *43*(1). https://doi.org/10.1042/BSR20220284

Feno, S., Munari, F., Gherardi, G., Reane, D. V., D'Angelo, D., Viola, A., Rizzuto, R., & Raffaello, A. (2023). Myonecrosis Induction by Intramuscular Injection of CTX. *Bio-Protocol*, *13*(1), e4587.

Fu, X., Li, S., Jia, M., Yang, W., & Hu, P. (2023). Induction of Skeletal Muscle Injury by Intramuscular Injection of Cardiotoxin in Mouse. *Bio-Protocol*, *13*(9), e4668.

Gama, J. F. G., Romualdo, R. D., de Assis, M. L., de Oliveira, L. M., Quírico-Santos, T., Alves, L. A., & Lagrota-Candido, J. (2022). Role of Regulatory T Cells in Skeletal Muscle Regeneration: A Systematic Review. *Biomolecules*, *12*(6). https://doi.org/10.3390/biom12060817

Guardiola, O., Andolfi, G., Tirone, M., Iavarone, F., Brunelli, S., & Minchiotti, G. (2017). Induction of Acute Skeletal Muscle Regeneration by Cardiotoxin Injection. *Journal of Visualized Experiments : JoVE*, *119*. https://doi.org/10.3791/54515

Hardy, D., Besnard, A., Latil, M., Jouvion, G., Briand, D., Thépenier, C., Pascal, Q., Guguin, A., Gayraud-Morel, B., Cavaillon, J.-M., Tajbakhsh, S., Rocheteau, P., & Chrétien, F. (2016). Comparative Study of Injury Models for Studying Muscle Regeneration in Mice. *PloS One*, *11*(1), e0147198.

Kapuscinski, J. (1995). DAPI: a DNA-specific fluorescent probe. *Biotechnic & Histochemistry : Official Publication of the Biological Stain Commission*, *70*(5), 220–233.

Kostrominova, T. Y. (2011). Application of WGA lectin staining for visualization of the connective tissue in skeletal muscle, bone, and ligament/tendon studies. *Microscopy Research and Technique*, *74*(1), 18–22.

Pizza, F. X., & Buckley, K. H. (2023). Regenerating Myofibers after an Acute Muscle Injury: What Do We Really Know about Them? *International Journal of Molecular Sciences*, *24*(16). https://doi.org/10.3390/ijms241612545

Rigamonti, E., Zordan, P., Sciorati, C., Rovere-Querini, P., & Brunelli, S. (2014). Macrophage plasticity in skeletal muscle repair. *BioMed Research International*, *2014*, 560629.

Wang, Y., Lu, J., & Liu, Y. (2022). Skeletal Muscle Regeneration in Cardiotoxin-Induced Muscle Injury Models. *International Journal of Molecular Sciences*, *23*(21). https://doi.org/10.3390/ijms232113380