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The Effects of An Extracellular Matrix Hydrogel on Muscle Pathology and Regeneration in Dystrophic Skeletal Muscle



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# The Effects of An Extracellular Matrix Hydrogel on Muscle Pathology and Regeneration in Dystrophic Skeletal Muscle

A dissertation submitted in partial satisfaction of the requirements for the degree Master of Science in Physiological Science

by

Kara Michelle Shin

2020

ABSTRACT OF THE THESIS

The Effects of An Extracellular Matrix Hydrogel

on Muscle Pathology and Regeneration

in Dystrophic Skeletal Muscle

by

Kara Michelle Shin

Master of Science in Physiological Science

University of California, Los Angeles, 2020

Professor Rachelle Hope Crosbie, Chair

In Duchenne muscular dystrophy (DMD), the lack of dystrophin leads to sarcolemma instability, which causes muscle to become fragile and waste over time. Biological scaffolds made of extracellular matrix (ECM) have been used in other disease models to promote tissue repair. This study investigates the effect of injecting porcine-derived ECM hydrogel on muscle pathology and regeneration in *mdx* mice, the murine model of DMD. We analyze common features of dystrophy including central nucleation, variable fiber cross-sectional area, and fibrosis. Previous work in our lab suggests that the failed regeneration observed in DMD is a result of inefficient cell adhesion and stem cell function caused by fibrotic ECM. Therefore, we examined cell adhesion by quantifying adhesion proteins laminin, utrophin, and dystroglycan. Because inflammation is also a barrier to stem cell function and biological scaffolds are known to polarize macrophage phenotypes, we also quantified macrophage markers to determine macrophage phenotype.

The thesis of Kara Michelle Shin is approved.

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University of California, Los Angeles

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## BACKGROUND

Duchenne muscular dystrophy (DMD) is a X-linked recessive disorder characterized by progressive muscle wasting and weakness. It is caused by mutations in the gene encoding for the protein dystrophin, which leads to the absence of the protein in individuals with DMD. Dystrophin is a critical component of the dystrophin-associated glycoprotein complex (DGC), a complex of proteins that attaches the actin cytoskeleton of myofibers to the surrounding extracellular matrix (ECM). The loss of dystrophin in DMD results in the loss of the DGC and subsequent instability of the sarcolemma, or muscle membrane [1-3]. Due to this sarcolemma instability, dystrophic muscle becomes fragile and susceptible to contraction-induced injury, resulting in muscle degeneration and atrophy [4, 5]. DMD is the most common, severe form of muscular dystrophy. Children born with DMD experience muscle weakness and degeneration, starting with the muscles of ambulation, and by their teenage years, they are often wheelchair reliant. Cardiac and diaphragm muscles are also affected in patients and can lead to cardiac or respiratory failure [6]. DMD is a lethal disorder that currently has no cure. Current treatments only help slow the progression of the disease.

The ECM is an essential and complex network of macromolecules, including collagen and laminin, present in all tissues [7]. It has an important role in the structural and biochemical support of resident cells [8]. In skeletal muscle, the ECM provides a mechanical framework that functions in force transmission and acts as a reservoir for stem cells, growth factors, and matrix remodeling enzymes. It is a dynamic structure that is degraded and replaced over time, and its remodeling is a critical part of tissue homeostasis. Previous studies have demonstrated the importance of a healthy ECM as a necessary microenvironment for proper muscle growth and repair [8, 9].

Recent studies have examined the potential of the ECM as a biological scaffold for tissue regeneration due to its diverse bioactive molecular content and its role in tissue homeostasis [9]. ECM scaffolds can be derived through decellularization of source tissues—small intestine submucosa, urinary bladder, cardiac muscle, skeletal muscle—and then implanted in the form of a scaffold sheet or injected in the form of a hydrogel to promote functional tissue remodeling. While the organization of ECM can vary between tissue types, ECM components are well-conserved among mammalian species, allowing these biological scaffolds to be well-tolerated by human hosts.

Biological scaffolds have been shown to help repair tissues in a number of models. For example, Rubin and colleagues showed formation of innervated, vascularized skeletal muscle islands following implantation of a scaffold into muscles of patients with volumetric muscle loss [10]. Christman's research group used an ECM hydrogel derived from porcine psoas muscle to improve muscle fiber size in ischemic tissues [11]. Lastly, Badylak and collaborators showed that the implantation of a scaffold small intestinal submucosa ECM into the abdomen of a rat resulted in formation of functional skeletal muscle with contractile force equal to that of the rat's native tissue [12]. The exact mechanism for how these ECM scaffolds facilitate tissue regeneration is currently unknown. The involvement of the ECM in providing a physical scaffold for muscle cells to bind to, in modulating macrophage phenotype, and in recruiting native progenitor cells are proposed mechanisms being studied [9].

Like native ECM, biological scaffolds provide both structural and biochemical support to the tissues they are implanted in [13]. They retain the structural and functional molecules secreted by the resident cells of their source tissues, and these components of the ECM, such as adhesion proteins and growth factors, are then provided to the host tissue when the scaffold is

implanted [9]. In healthy skeletal muscle, tissue regeneration following injury or exercise relies on communication between resident cells and their native ECM [9]. Similarly, these biological scaffolds modulate behavior of the cells they come into contact with and can influence tissue phenotype [13]. Previous studies using ECM scaffolds have shown that scaffold-treated muscles show a pro-remodeling macrophage phenotype, which is associated with muscle repair and growth [9, 11, 14, 15].

In *mdx* mice, the murine model for DMD, adhesion proteins laminin and utrophin have been hypothesized to help stabilize the sarcolemma as a compensatory mechanism. Instead of a DGC at the muscle membrane, the utrophin-associated glycoprotein complex (UGC) connects the ECM to the muscle cytoskeleton in *mdx* tissue [16]. Utrophin is a structural and functional paralogue of dystrophin [17]. It binds actin in the muscle fiber and alpha-dystroglycan, a protein in the UGC that connects to laminin through beta-dystroglycan. Laminin is the primary binding partner for the UGC in the ECM. In healthy skeletal muscle, laminin is also responsible for binding the DGC to connect the complex to the ECM. Loss of specific isoforms of laminin are associated with other muscular dystrophies [18].

In this study, we examine the effects of a porcine-derived ECM hydrogel on muscle pathology and regeneration in *mdx* mice [2]. There are two specific aims for this paper. Our first aim is to test the hypothesis that providing a matrix hydrogel will improve muscle pathology in *mdx* mice. We examine the effects of the hydrogel on muscle pathology by looking at common markers of pathological regenerating skeletal muscle, including central nucleation, myofiber cross-sectional area, and fibrosis. Our second aim examines possible mechanisms for how a matrix hydrogel affects muscle pathology. We test the hypothesis that the hydrogel improves cell adhesion complexes at the muscle membrane by increasing the abundance of laminin and

components of the UGC. Work in our lab has demonstrated that fibrotic ECM present in skeletal muscle from a DMD mouse model prevents efficient adhesion and function of muscle progenitor cells and muscle stem cells. These data suggest that treatments improving cell adhesion have potential for preventing failed regeneration of skeletal muscle and promoting tissue remodeling. We also test the hypothesis that the hydrogel polarizes the macrophage phenotype. By providing this matrix substrate, we hope to alter the environment in dystrophic muscle to promote healthy muscle remodeling.

# CHAPTER 1: IMPACT OF A MATRIX HYDROGEL ON MUSCLE PATHOLOGY

## Introduction

While healthy skeletal muscle fibers have peripherally located nuclei, dystrophic muscle fibers have centrally located nuclei resulting from chronic cycles of degeneration and regeneration [19, 20]. Central nucleation is a sign of muscle undergoing regeneration. When activated muscle stem cells fuse with a damaged fiber, a new nucleus is incorporated in the center of the regenerating fiber before it is moved to the periphery [21]. Because muscle degeneration in DMD is a result of damage to the sarcolemma and the hydrogel should limit this damage by improving cell adhesion and stabilizing the sarcolemma, we hypothesized that there will be a lower percentage of centrally nucleated fibers in hydrogel-injected tissues, indicating there are less cycles of degeneration and impaired regeneration occurring.

Another difference in muscle architecture between dystrophic and healthy skeletal muscle is the variation in muscle fiber size. Healthy, adult skeletal muscle typically has uniform cross-sectional area. Skeletal muscle from *mdx* tissue is known to have less uniform cross-sectional area of myofibers when compared to wild-type tissue [22]. This is due to the presence of larger hypertrophic fibers as well as clusters of small newly regenerating fibers. When muscle fibers experience necrosis, skeletal muscle attempts to repair itself and newly regenerating fibers have smaller cross-sectional area than normal muscle fibers. Due to the ongoing degeneration in DMD, dystrophic muscle is often composed of pools of these smaller fibers [22]. The variable fiber cross-sectional area observed in dystrophic skeletal muscle is also a result of the regeneration following necrosis. Because the hydrogel is hypothesized to improve cell adhesion to stabilize the sarcolemma, we predict that hydrogel-injected tissues will have more uniformly sized fibers.

A common pathological feature of DMD is fibrosis or excess ECM deposition, which is a result of the chronic cycles of degeneration and regeneration. While controlled ECM deposition functions as a scaffold for newly developing tissue during muscle growth and repair, excess ECM deposition is pathological because it can reduce both muscle function and the amount of muscle tissue available for repair [23]. Data from our lab has shown that fibrotic regions in *mdx* tissue impair cell adhesion and function of muscle progenitor cells, which could lead to failed muscle regeneration in DMD. We hypothesize that there will be less fibrosis in hydrogel-injected tissues. To examine the effects of the hydrogel on fibrosis, we quantified the major fibrillar ECM protein, collagen I, which is known to be increased in *mdx* mice when compared to wild type mice.

## Materials and Methods

### Animals

Nine male *mdx* mice at 4-weeks-old were used for these experiments. All mice were originally obtained from Jackson Laboratory, bred, and maintained in our animal facility.

### Skeletal Muscle Decellularization and Hydrogel Preparation

The ECM hydrogel used in these experiments was generated by Dr. Karen Christman's laboratory at UCSD [24]. Porcine skeletal muscle was decellularized, then lyophilized and milled into a fine powder. This powder was then enzymatically digested into a liquid, which assembles into a hydrogel when exposed to physiological pH and temperatures. The concentration of the solubilized hydrogel is 6 mg/ml.

### Hydrogel Treatment

We injected the left tibialis anterior muscle of 9 male *mdx* mice at four-weeks-old with 18 μL hydrogel (n=6) or PBS (n=3). Each injection was given in the center of the muscle belly

with an insulin syringe. Contralateral TAs of each animal were left alone. All 9 mice were sacrificed, and their TAs were harvested 5-weeks post-injection. All TA muscles once harvested were mounted in OCT (Tissue-Tek) and flash frozen with liquid nitrogen-cooled isopentane. Frozen TA tissues were then stored in a -80°C freezer.

Indirect Immunofluorescence

Cross-sections of TA muscles were sectioned at -22°C at a thickness 30 μm. 30 μm sections from tissues harvested 5 weeks post-injection were placed on slides with tissue tack (Electron Microscopy Sciences, Hatfield, PA, USA) and decellularized in 1% SDS for 30 minutes, followed by 30-minute rinses in PBS, di$H_2O$, and PBS, consecutively. Decellularized tissue sections were then stained for collagen type I using primary antibody anti-mouse collagen type I produced in rabbit (1:200, Cedarlane, CL50151AP).

Imaging and Fluorescence Quantification

Tissues were imaged using Zeiss Axio Imager M2 microscope and Zen blue edition software. 30 um tissue sections stained for collagen I were imaged at 10x objectives and 20x objectives. Zen Blue edition software was used to quantify fluorescence intensities of collagen stain. The program calculates mean intensity values by measuring the frequency of EGFP per fluorescence intensity. Fluorescence was quantified from a whole cross-section image of collagen-stained tissue. For each animal, 2 images or cross-sections were analyzed, each taken from a different level of the muscle.

Histology and Central Nucleation

10 μm, intact muscle sections were stained with hematoxylin and eosin and analyzed in ImageJ using the Cell Counter plugin. Myofibers with central nucleation and myofibers with

peripheral nucleation were counted. The percentage of centrally nucleated fibers were calculated as follows:

$$\frac{Number\ of\ Centrally\ Nucleated\ Fibers}{Total\ Number\ of\ Muscle\ Fibers} \times\ 100\% \ = \%\ CNF$$

Cross-Sectional Area

30 μm sections that were decellularized and stained for laminin using an immunofluorescence assay were analyzed in ImageJ. Laminin staining was used to mark boundaries of individual myofibers. Cross-sectional area measurements of myofibers were measured using a macro plugin provided by Dr. Karen Christman's Laboratory at UCSD. Myofibers that were not recognized by plugin were manually circled in ImageJ.

Statistical Analyses & Figures

Graphs and histograms were made using GraphPad Prism 8 software. Statistical tests were performed also using Prism. Figures were generated in Microsoft PowerPoint.

**Results**

Muscle fibers from *mdx* mice have centrally located nuclei as a result of continuous degeneration and impaired regeneration. To visualize central or peripheral nucleation, we stained both PBS-treated and hydrogel-treated TAs with hematoxylin and eosin (Figure 1A). The number of fibers with central or peripheral nucleation in a cross-section were manually counted. Three animals were excluded due to tissues being freeze fractured. Quantification showed there is no difference in the percentage of centrally nucleated fibers between control and hydrogel-treated TAs (Figure 1B). *Mdx* mice are also known to have less uniform myofiber cross-sectional area when compared to wild-type mice. To analyze myofiber cross-sectional area, we stained TA sections for ECM protein laminin, which allowed us to determine the myofiber boundary. Visual analysis and quantification showed there is no difference in mean cross-sectional area between

control and hydrogel-treated TAs (Figure 2A-B). Distribution of fiber cross-sectional area measurements were also similar between groups. Both hydrogel-treated and PBS-treated tissues showed a greater proportion of smaller fibers than larger fibers, and we observed variable fiber cross-sectional area for both hydrogel-treated and PBS-treated muscles (Figure 2C).

Fibrosis is a hallmark of DMD. To examine the effect of the hydrogel injection on fibrosis, we stained for ECM protein collagen I, which is known to contribute to fibrosis in *mdx* mice (Figure 3A). Fluorescence was quantified from a whole cross-section image of collagen-stained tissue. For each animal, 2 images or cross-sections were analyzed, each taken from a different area of the muscle. Although there was not a significant difference, hydrogel-treated tissues trended toward having a lower mean corrected total fluorescence for collagen I compared to PBS-treated tissues, suggesting the hydrogel may be modulating fibrosis in *mdx* muscle (Figure 3B).

**Discussion**

In DMD, as a result of cycles of necrosis and impaired regeneration, dystrophic muscle fibers have centrally located nuclei rather than peripherally located nuclei. We hypothesized that the hydrogel-injected tissues would have less central nucleation than control tissues. We observed that there was no difference in the percentage of centrally nucleated fibers between hydrogel-injected tissues and PBS-injected tissues. These results suggest that the hydrogel is not preventing the cycles of degeneration and regeneration. Because central nucleation is only a sign of ongoing regeneration, definitive conclusions on if the hydrogel is improving the impaired regeneration cannot be drawn.

Another indicator of muscle regeneration is variable cross-sectional area of myofibers, which is also observed in DMD [22, 25]. While wild-type fibers have uniform cross-sectional

area, *mdx* fibers have variable cross-sectional area due to the presence of pathological hypertrophic fibers and smaller regenerating fibers. Quantification of muscle fiber cross-sectional area showed that myofiber size was not different between hydrogel-treated and PBS-treated tissues. These results aligned with our quantification of central nucleation and imply that the hydrogel does not prevent cycles of degeneration and regeneration. The distribution of fiber size was also similar between hydrogel-injected and PBS-injected muscles. Both groups exhibited variable fiber cross-sectional area and had a greater portion of smaller fibers , which further supports that ongoing regeneration is occurring.

While the hydrogel did not significantly alter the pathology of *mdx* tissues, initial analysis of collagen I demonstrated that the hydrogel may influence fibrosis, a hallmark of DMD. As discussed above, functional muscle tissue is lost and replaced by fatty and fibrotic tissue in DMD. This is a result of chronic inflammation that signals fibroblasts and myofibroblasts to produce ECM. Excess ECM stimulates fibroblasts to further increase ECM production, and increased fiber production without reciprocally balanced degradation leads to overaccumulation of the ECM, or fibrosis [26] . Work in our lab has shown that in DMD, this fibrotic ECM impairs muscle regeneration by impeding muscle progenitor cell function, including cell adhesion and cell differentiation. This study showed that hydrogel-injected tissues trended towards having less collagen I than PBS-injected tissues, suggesting that the hydrogel can help limit fibrosis. However, variability in collagen I distribution might be diluting any quantifiable difference and thus additional mice and more specific quantification, such as collagen I thickness, may be required.

By introducing the hydrogel to the muscle microenvironment, we aimed to provide the injected *mdx* tissue with a functional and biologically active ECM that promotes healthy resident

cell function. In healthy muscle, ECM remodeling, which involves protein degradation and synthesis, occurs in response to injury or exercise [26]. ECM remodeling is a tightly regulated, coordinated process that involves communication between muscle cells and the ECM [27]. Disrupting the ECM remodeling process can lead to harmful consequences for resident cells [7]. Abnormal ECM dynamics, where there is an imbalance between ECM protein degradation and synthesis, are responsible for pathology in other diseases, such as pulmonary fibrosis and cancer [28].

In DMD, excess ECM deposition contributes to the muscle pathology and promotes muscle dysfunction. Future experiments can further examine the potential benefits of the hydrogel for limiting fibrosis by looking at its effects on fibroblast activity. Fibroblasts are the cells responsible for producing collagen in skeletal muscle [29]. They also secrete matrix metalloproteinases, the primary enzymes responsible for ECM degradation [7]. We can culture fibroblasts on hydrogel-injected and PBS-injected *mdx* tissues to observe their interaction with the ECM. By examining the role of muscle fibroblasts in ECM protein synthesis and degradation, we can test the hypothesis that the breakdown of the biological scaffold improves ECM dynamics in *mdx* skeletal muscle. Investigating the mechanism of abnormal ECM dynamics, specifically the imbalance between ECM protein synthesis and degradation, occurring in *mdx* tissue may allow for alternative therapies targeting fibrosis. Promoting protease-mediated degradation of the ECM could be a potential mechanism for preventing fibrosis and improving muscle pathology in DMD.

# CHAPTER 2: IMPACT OF A MATRIX HYDROGEL ON CELL ADHESION AND MACROPHAGE PHENOTYPE

## Introduction

In healthy skeletal muscle, dystrophin strengthens the sarcolemma by linking the cytoskeleton of muscle fibers to the surrounding ECM. While actin connects the DGC to the muscle fiber, laminin binds the DGC in the ECM. Binding of the DGC to both actin and laminin provides a mechanical link for force transmission in skeletal muscle. In DMD, the loss of dystrophin and the DGC causes muscle to lack this important mechanical link between actin and the ECM. Without it, muscle experiences contraction-induced injury, which over time leads to necrosis of muscle fibers.

*Mdx* mice have increased levels of laminin and this is hypothesized to be a compensatory mechanism to help stabilize the sarcolemma [30]. Certain forms of laminin are also associated with protection against the contraction-induced injury that occurs in dystrophic muscle [30, 31]. Instead of a DGC at the muscle membrane, the utrophin-associated glycoprotein complex (UGC) connects the ECM to the muscle cytoskeleton in *mdx* tissue [16]. Utrophin is a structural and functional paralogue of dystrophin [17]. While utrophin is typically confined to the neuromuscular junctions, myotendinous junctions, and blood vessels in healthy, adult skeletal muscle, the protein is increased at the sarcolemma as a compensatory mechanism in dystrophic muscle [32, 33].

In this chapter, we investigated potential mechanisms for how the hydrogel could benefit the muscle niche. Previous work in our lab suggests that the failed regeneration observed in DMD is a result of inefficient cell adhesion and stem cell function caused by fibrotic ECM. Therefore, we examined cell adhesion by quantifying adhesion proteins laminin, utrophin, and

dystroglycan. Because the hydrogel matrix used in our experiments retains the composition of the skeletal muscle ECM it is derived from, introduction of the hydrogel to the muscle niche has the potential to increase the abundance of available laminin for binding of the UGC and other cell adhesion complexes to promote sarcolemma stabilization. We hypothesized that hydrogel-injected tissues would have greater levels of laminin. Because utrophin is linked to laminin through dystroglycan, we also examined alpha-dystroglycan and beta-dystroglycan. We hypothesized that these components of the UGC would be increased in hydrogel-injected tissues as a result of the increased laminin available for the UGC to bind.

In DMD, a large barrier to tissue remodeling is the infiltration of specific immune cells that exacerbate muscle repair and regeneration. While the immune response is an important step in tissue regeneration, certain populations of macrophages do not support successful regeneration and these populations are known to contribute to the *mdx* muscle pathology [34]. Macrophages can be classified as M1 or M2, where M1 macrophages are pro-inflammatory and are associated with tissue damage and M2 macrophages are anti-inflammatory and promote tissue repair [35]. When there are more M2 macrophages present in the immune cell population, the phenotype is M2, also known as the pro-remodeling phenotype.

The polarization of macrophage phenotype is one proposed mechanism for how biological scaffolds promote tissue repair in their hosts. Wagner and colleagues have shown that the injection of a porcine-derived hydrogel can increase the presence of M2 macrophages in a given population of inflammatory cells observed in *mdx* mice [36]. Their study examined the effects of a hydrogel-myostatin co-delivery on the population of inflammatory cells recruited. Our study investigates macrophage phenotype as an indicator for successful ECM remodeling outcomes. Because other ECM scaffolds have been observed to promote a M2 macrophage

phenotype, we examined the effect of the hydrogel on macrophage phenotype in *mdx* mice and hypothesized that the hydrogel will recruit more M2 macrophages and shift the population to an anti-inflammatory, pro-remodeling phenotype.

**Materials and Methods**

Animals

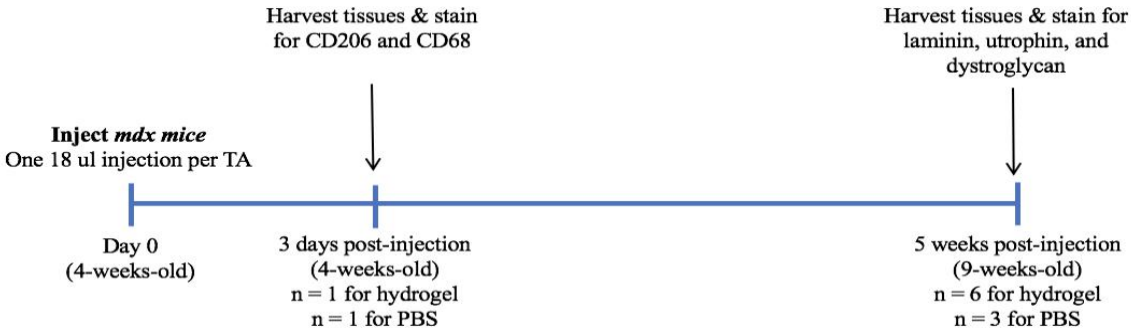
11 male *mdx* mice at 4-weeks-old were used for these experiments. All mice were originally obtained from Jackson Laboratory, bred, and maintained in our animal facility.

Skeletal Muscle Decellularization and Hydrogel Preparation

The ECM hydrogel used in these experiments was generated by Dr. Karen Christman's laboratory at UCSD. Porcine skeletal muscle was decellularized, then lyophilized and milled into a fine powder. This powder was then enzymatically digested into a liquid, which assembles into a hydrogel when exposed to physiological pH and temperatures. The concentration of the solubilized hydrogel is 6 mg/ml.

Hydrogel Treatment

We injected the left tibialis anterior muscle of 11 male *mdx* mice at four-weeks-old with 18 μL hydrogel or PBS. Each injection was given in the center of the muscle belly with an insulin syringe. Contralateral TAs of each animal were left alone. For accessing macrophage phenotype, 2 of the injected mice were sacrificed, and their TAs harvested 3-days post injection. For all other experiments, the remaining 9 mice were sacrificed, and TAs harvested 5-weeks post-injection (see timeline below). All TA muscles once harvested were mounted in OCT (Tissue-Tek) and flash frozen with liquid nitrogen-cooled isopentane. Frozen TA tissues were then stored in a -80° Celsius freezer.




Indirect Immunofluorescence Assay

Cross-sections of TA muscles were cut at -22°C at a thickness of 10 or 30 μm lengths. 10 μm intact muscle sections from tissues harvested 3 days post-injection (n=2) were stained for M1 and M2 macrophage markers CD68 (1:100, BioRad, MCA1957) and CD206 (1:50, BioRad, MCA2235), respectively. 10 μm intact muscle sections from tissues harvested 5 weeks post-injection (n=9) were stained for cell adhesion proteins—utrophin, alpha-dystroglycan, and beta-dystroglycan—using primary antibodies Mancho3 (1:5, DSHB, MANCHO3(8A4)), IIH6 (1:25, SCBT, sc-53987), and Mandag2 (1:50, DSHB, MANDAG2(7D11)), respectively. 30 μm sections from tissues harvested 5 weeks post-injection (n=9) were placed on slides with tissue tack and decellularized in 1% SDS for 30 minutes, followed by 30-minute rinses in PBS, di$H_2O$, and PBS, consecutively. Decellularized tissue sections were then stained for laminin using primary antibody anti-laminin produced in rabbit (1:200, Sigma, L9393-100UL).

Imaging and Fluorescence Quantification

Tissues were imaged using Zeiss Axio Imager M2 microscope and Zen blue edition software. 10 um tissue sections stained for macrophage markers were imaged at 10x objectives. 30 um tissue sections stained for laminin as well as 10 um sections stained for adhesion proteins were imaged at 10x objectives and 20x objectives. Zen Blue edition software was used to

quantify fluorescence intensities of protein stain. The program can measure mean intensity values by measuring frequency of EGFP per fluorescence intensity. Fluorescence was quantified from a whole cross-section image when measuring abundance of laminin, utrophin, alpha-dystroglycan, and beta-dystroglycan. For each animal, 2 images or cross-sections were analyzed, each taken from a different level of the muscle.

Statistical Analyses & Figures

Graphs and histograms were made using GraphPad Prism 8 software. T-tests were performed also using Prism. A priori analysis for sample size estimation was performed using G*Power Analysis. Figures and injection timeline graphics were generated in Microsoft PowerPoint.

**Results**

To quantify cell adhesion, we stained for laminin and components of the UGC including utrophin, alpha-dystroglycan, and beta-dystroglycan. For each protein analyzed, we obtained two sections taken from separate regions of the TA. Quantification of the normalized mean intensity of laminin indicated no difference between control and hydrogel-treated groups with a wide range of variability of laminin deposition (Figure 1A-B). We observed that hydrogel-treated muscles had a greater mean intensity of utrophin than PBS-treated muscles (Figure 1C). There was no difference in mean intensity of alpha-dystroglycan or beta-dystroglycan between hydrogel-treated and PBS-treated muscles (Figure 1D-E).

To determine macrophage phenotype, we stained serial muscle sections for M1 and M2 macrophage markers CD68 and CD206, respectively. CD68 is highly expressed by monocytes and macrophages and is known as a pan macrophage marker that can identify M1 and M2 macrophages [34]. CD206, also known as mannose receptor, is a cell surface receptor expressed

on macrophages. It is commonly used to identify M2 macrophages [15]. Because immune cell infiltration is variable, we quantified cells in three separate cross-sections taken at different levels of the muscle. We observed the ratio of CD206-positive cells to CD68-positive cells was greater in the hydrogel-injected muscle. (Figure 2A-B). The hydrogel-treated TA also trended toward a smaller population of M1 cells and a greater population of M2 cells on average (Figure 2C).

**Discussion**

We observed that hydrogel-treated muscles had a higher mean intensity of utrophin. Utrophin has been hypothesized to compensate for dystrophin in dystrophin-deficiencies [33, 37]. Through the assembly of the UGC, utrophin can facilitate binding of the actin cytoskeleton in muscle to the surrounding ECM and help stabilize the sarcolemma. We anticipated that levels of utrophin would be increased in hydrogel-treated tissues as a result of the hydrogel providing more laminin available to bind the UGC in the muscle microenvironment. A greater abundance of utrophin and dystroglycan in hydrogel-injected tissues would suggest that introducing the hydrogel to *mdx* tissue increases the UGC at the sarcolemma, which is beneficial for dystrophic muscle. However, while we did observe greater levels of utrophin in the hydrogel-treated muscle, we did not observe a difference in levels of laminin, alpha-dystroglycan, or beta-dystroglycan between hydrogel-treated and PBS-treated tissues.

Similar to our collagen I staining, variability in distribution of laminin, alpha-dystroglycan, and beta-dystroglycan might be diluting any quantifiable differences, and thus more specific quantification may be required. It is possible that the mean intensity of laminin was not representative of the abundance of laminin. In a single muscle cross-section, there are areas with increased laminin deposition and areas with less. Quantification of the intensity of

laminin around different groups of myofibers may represent the abundance of laminin following hydrogel injection more accurately. It is also possible that staining with a pan-laminin antibody prevented us from seeing changes occurring for distinct laminin isoforms. Specific laminin isoforms have been hypothesized to stabilize the sarcolemma. Burkin's research group demonstrated that treating *mdx* mice with laminin-111 protected them from exercise-induced injury [30]. Laminin-211 is the most abundant form of laminin in skeletal muscle and is essential for muscle integrity [31]. Mutations in laminin-211 leads to congenital muscular dystrophy [18]. Staining with an antibody for laminin-211 rather than a pan-laminin antibody may show more robust differences. In addition to alternative methods for quantifying laminin, increasing the sample size may also be required. A priori analysis estimates at least 21 hydrogel-treated animals are needed to acquire a statistically significant difference for mean intensity of laminin (alpha =.05).

Macrophage phenotypes have been used as indicators of remodeling outcomes following degradation of implanted biological scaffolds [38]. We were interested in examining macrophage phenotype as an indicator for our hydrogel-injected tissues. Our initial quantification showed a hydrogel-treated muscle had a greater ratio of M2 cells to M1 cells than a PBS-treated muscle. This result indicates a M2 phenotype associated with muscle repair and growth in skeletal muscle. However, more tissues need to be analyzed for definitive conclusions on the effect of our porcine-derived hydrogel on macrophage phenotype in *mdx* mice. The results of our initial quantification also align with current literature on the hydrogel's ability for macrophage polarization. Jin and colleagues observed that a scaffold derived from porcine cardiac muscle could both decrease the amount of pro-inflammatory macrophages and increase the number of anti-inflammatory macrophages in a rat model of volumetric muscle loss [14]. Our hydrogel-

injected muscle had less M1 cells and more M2 cells compared to the PBS-treated muscle. If the hydrogel can recruit the appropriate immune cells that promote skeletal muscle remodeling, then polarization would be beneficial to dystrophic muscle where inflammation is a major contributor to the muscle pathology.

In both this study and previous pilot studies conducted in our lab, the hydrogel has shown potential for increasing abundance of utrophin in *mdx* mice. These results show the potential of the hydrogel for improving cell adhesion in dystrophic muscle. We can further examine the hydrogel's effect on cell adhesion by investigating membrane stability. Because loss of the DGC causes the sarcolemma instability, we anticipate that promoting cell adhesion will limit damage to the sarcolemma. We can test the hypothesis that the hydrogel promotes membrane stability in *mdx* mice by staining for proteins IgG and IgM. IgG and IgM are large serum proteins that cannot cross an intact plasma membrane. Detection of these proteins inside the muscle fiber indicates disruptions of the sarcolemma. By staining for these different size proteins, we can access the extent of membrane integrity for hydrogel-injected and PBS-injected tissues.

Additional experiments examining the effect of the hydrogel on cell adhesion and macrophage phenotype in *mdx* mice must be conducted before definitive conclusions can be made. If the hydrogel can increase cell adhesion and recruit immune cells that are anti-inflammatory and pro-remodeling, then it shows promising potential for improving the satellite cell niche in *mdx* mice. Maintaining tissue homeostasis in skeletal muscle depends on the ECM's interactions with satellite cells, which are muscle-specific stem cells [39]. Adult stem cells are responsible for generating new tissue in response to injury or disease. While inflammatory cytokines released by an injury can activate satellite cells, chronic inflammation can augment satellite cell function [40]. In DMD, the regenerative capacity of satellite cells become exhausted

and this dysfunction contributes to the failed regeneration [41]. To examine if the hydrogel facilitates muscle regeneration, we propose to culture satellite cells on hydrogel-injected tissues and observe the differentiation of these stem cells. While our central nucleation and cross-sectional area analyses suggest that our tissues undergo regeneration, those markers did not provide further detail on the type of regeneration occurring. This experiment would test the hypothesis that the hydrogel promotes healthy regeneration in dystrophic muscle by influencing satellite cell activity. Future studies that investigate the effects of the hydrogel on the muscle microenvironment would allow us to better understand the role of the ECM in *mdx* muscle pathology and the needs of resident cells for a healthy muscle niche.

## CONCLUSION

The results of this study suggest that the hydrogel has the potential to improve cell adhesion in *mdx* skeletal muscle. However, while we did observe higher levels of utrophin in hydrogel-injected tissues than PBS-injected tissues, we saw no difference in levels of laminin, alpha-dystroglycan, or beta-dystroglycan between hydrogel-treated and PBS-treated muscles. We consistently observed variability in the distribution of these proteins, so further research with more specific quantification are needed to confirm that these results were an accurate representation of the hydrogel's impact on these specific proteins. A larger sample size may also be required to show any robust differences. Further analysis of macrophage markers is also required before any conclusions can be made about the impact of the hydrogel on macrophage phenotype in *mdx* mice.

We did not see improvements in *mdx* muscle pathology. The identification of central nucleation and variable cross-sectional area in hydrogel-injected tissues illustrates that hydrogel-treated muscles still undergo continuous regeneration. These results raise the question on whether the hydrogel is affecting the type of regeneration occurring in *mdx* mice. Because native ECM plays a large role in directing stem cell activity, we anticipate that the ECM-derived hydrogel has the potential to promote a healthy stem cell niche and thus, promote healthy skeletal muscle regeneration. Future directions will investigate the hydrogel's ability to 1) provide a matrix substrate for cell adhesion complexes to bind, and 2) promote tissue remodeling by influencing satellite cells in *mdx* mice. These studies would also advance our understanding of the role of the ECM in *mdx* muscle pathology.

## FIGURES

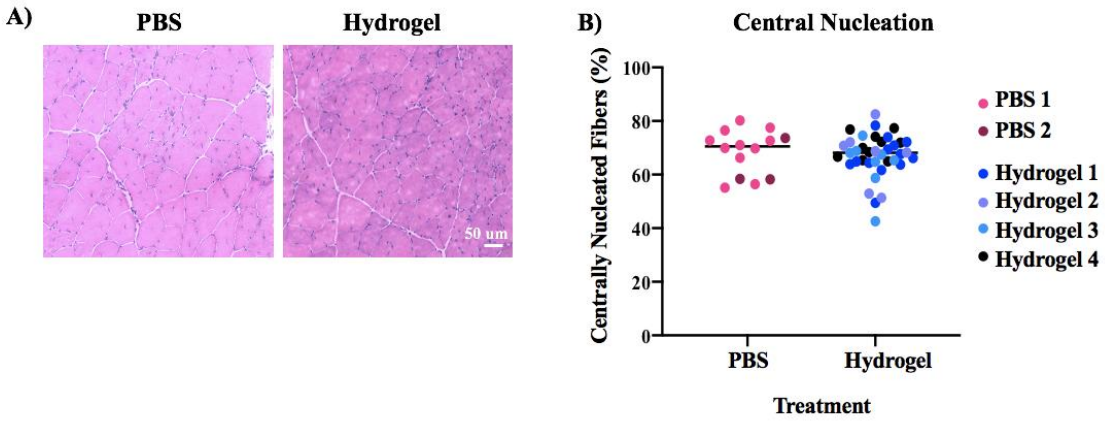



**Figure 1-1. Hydrogel Does Not Change Percentage of Centrally Nucleated Fibers.** 10 μm tibialis anterior cross-sections were stained with hematoxylin and eosin (A) to visualize central or peripheral nucleation. (B) There is no difference in percentage of centrally nucleated muscle fibers between PBS-treated (n=2) and hydrogel-treated (n=4) groups. Group differences were analyzed by t-test.

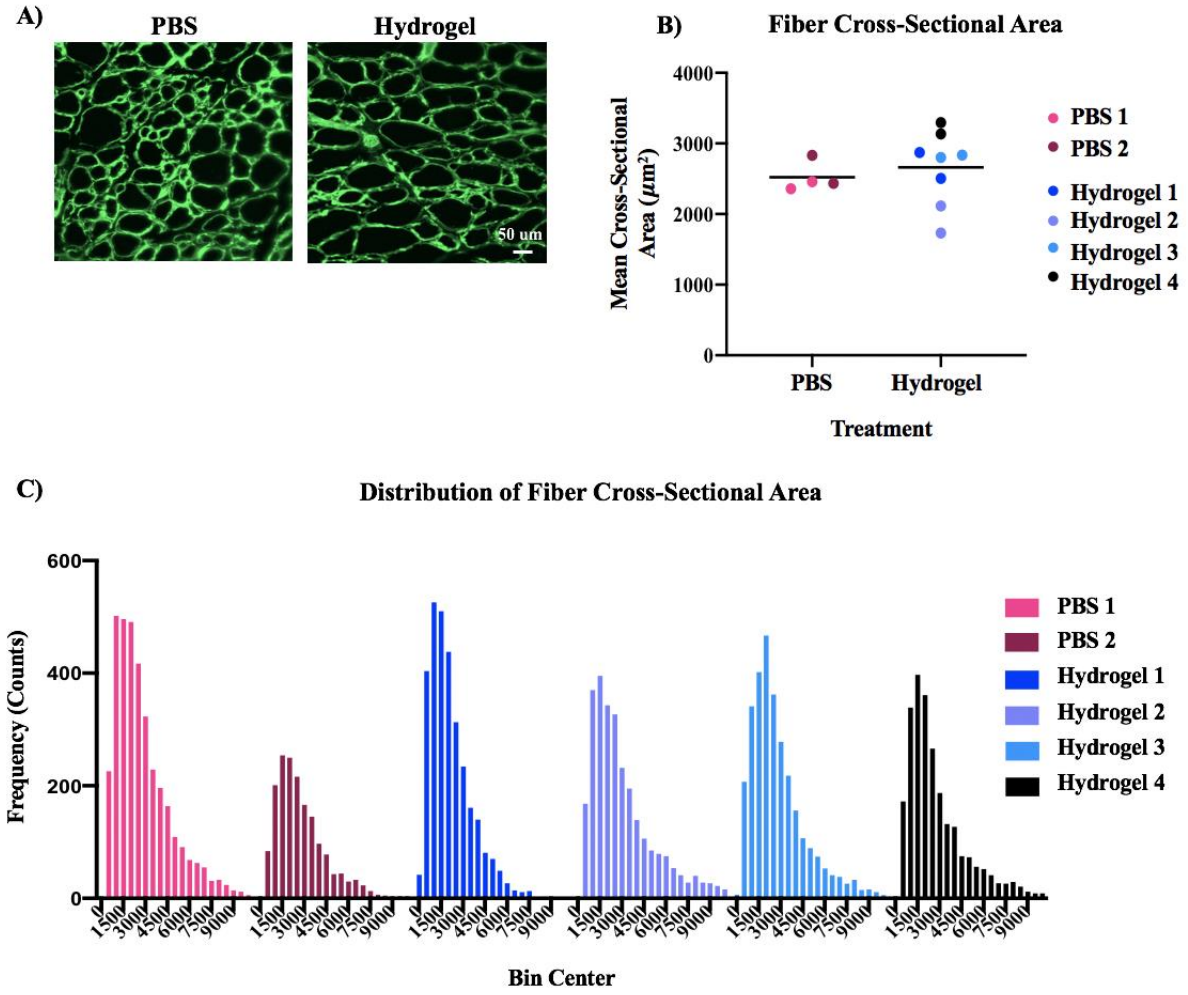



**Figure 1-2. Hydrogel Does Not Change Fiber Cross-Sectional Area.** (A) 30 μm tibialis anterior sections of hydrogel-injected (n=4) and PBS-injected (n=2) muscles were decellularized and then stained for laminin. (B) There is no difference in mean cross-sectional area between PBS-treated and hydrogel-treated muscle fibers. Group differences were analyzed by t-test. (C) The distribution of fiber size was similar between groups with a greater proportion of smaller fibers, and both groups show variable fiber cross-sectional area.

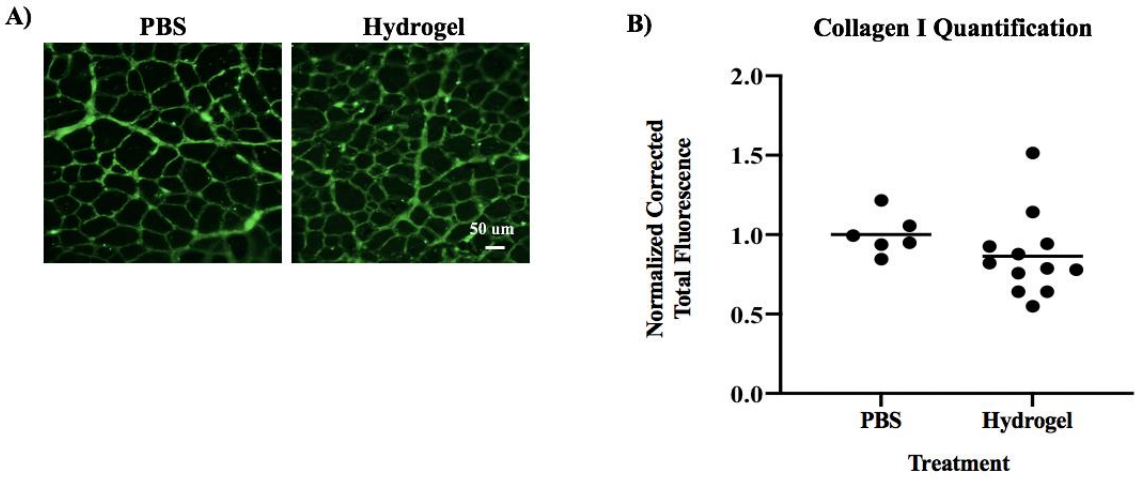



**Figure 1-3. Trend of Decreased Fibrosis in Hydrogel-Injected Tissues.** (A) 30 μm tibialis anterior sections were decellularized and then stained for collagen I, a major ECM protein that contributes to fibrosis. (B) There is a trend toward PBS-treated (n=3) muscle fibers having more collagen I than hydrogel-treated (n=6) muscle fibers. Group differences were analyzed by t-test.

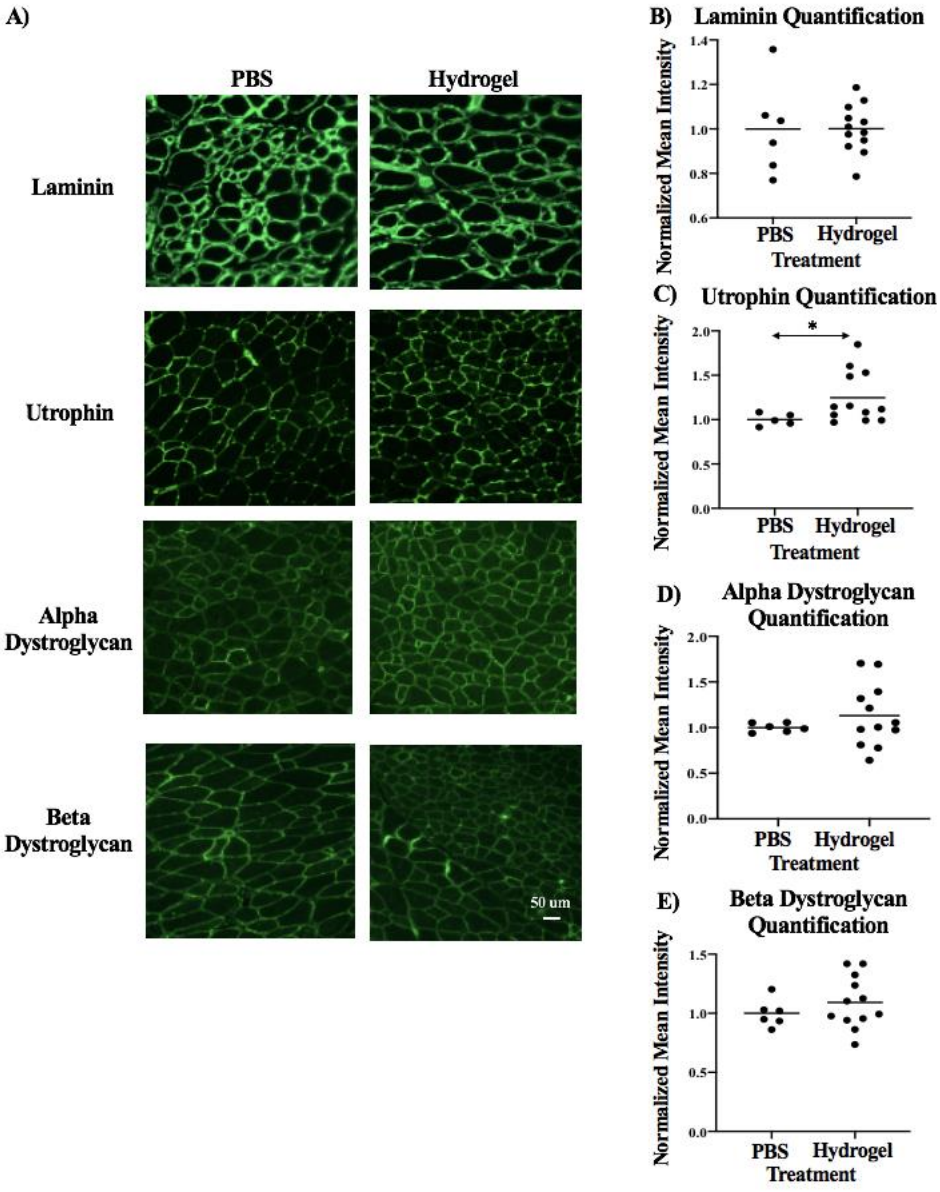



**Figure 2-1. Effect of Hydrogel Injection on Cell Adhesion Proteins.** (A) 30 μm tibialis anterior sections from hydrogel-treated (n=6) and PBS-treated mice (n=3) were decellularized and then stained for laminin. 10 μm tibialis anterior sections were stained for proteins utrophin, alpha-dystroglycan, and beta-dystroglycan. (B) There is no difference in mean intensity for laminin between PBS-treated and hydrogel-treated muscle fibers. (C) There is an increase in mean intensity of utrophin for hydrogel-treated fibers than PBS-treated fibers. Group differences analyzed by t-test with Welch's correction ($P$ =.0155). (D) There is no difference in mean intensity of alpha-dystroglycan between PBS-treated and hydrogel-treated muscle fibers. (E). There is no difference in mean intensity of beta-dystroglycan for PBS-treated and hydrogel-treated tissues. Statistical analyses were performed for all assays.

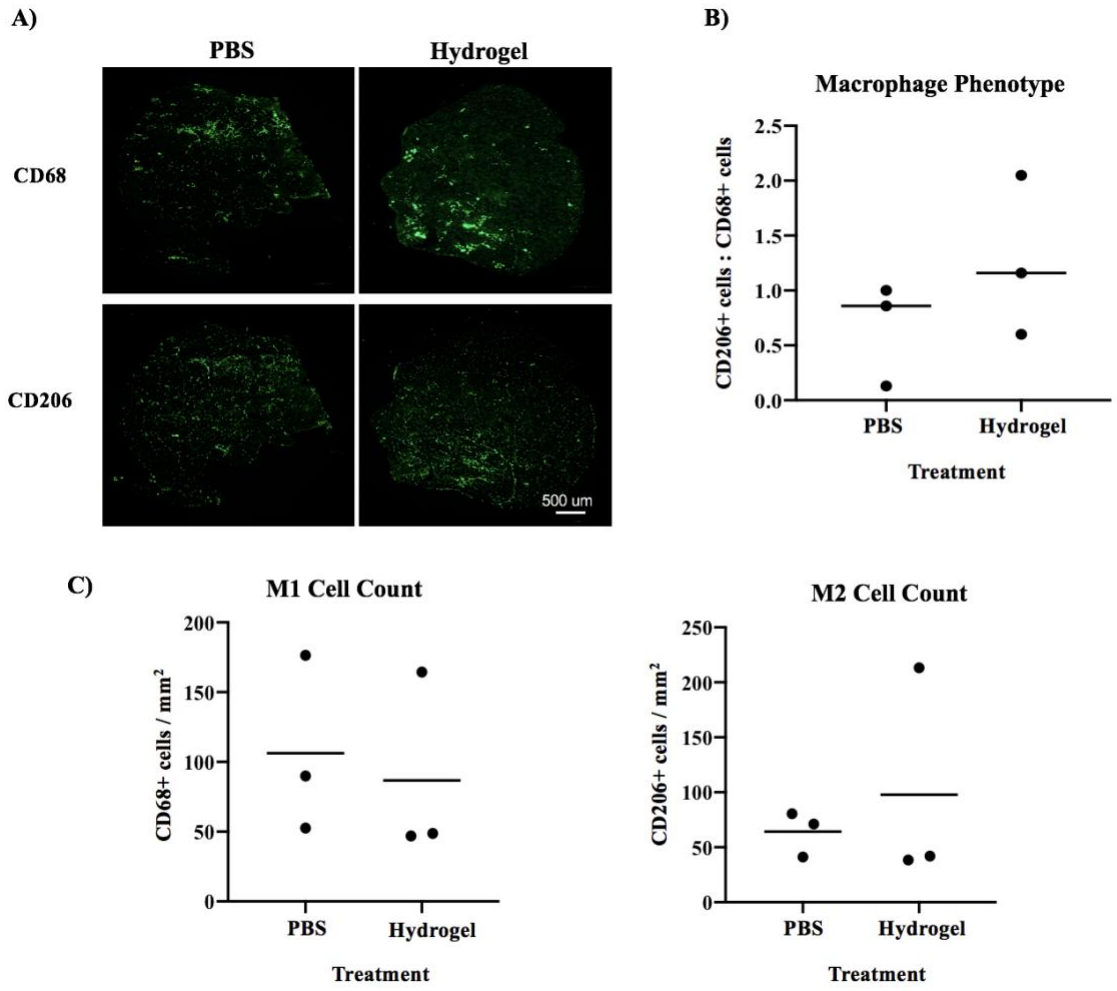



**Figure 2-2. Hydrogel Potentially Shifts Macrophage Phenotype to Pro-Remodeling Phenotype.** (A) 10 μm serial tibialis anterior sections were stained for M1 macrophage marker CD68 or M2 macrophage marker CD206. (B) There was a shift to M2 phenotype for the hydrogel-treated animal (n=1) when compared to the PBS-treated animal (n=1). (C) This shift involves a decrease in M1 cell population and an increase in the M2 cell population.

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