Regulation of Inflammation and Skeletal Muscle Repair by Mohawk and Eosinophils

by

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ABSTRACT

Skeletal muscle injury, whether acute or chronic, is characterized by influxes of pro- and anti-inflammatory cells that coordinate with muscle to precisely control the reparative process. This intricate coordination is facilitated by a signaling feedback loop between satellite cells and extravasated immune cells. Regulation of the cytokines and chemokines that mediate healthy repair is critical for the overall success of fiber regeneration and thus provides a prospective direction for the development of therapeutics aimed at fine-tuning the local inflammatory response. This work describes (1) the contribution of non-myogenic cells in skeletal muscle regeneration, (2) the role of the transcription factor Mohawk (Mkx) in regulating inflammation following acute muscle injury and the identification of an overarching requirement for Mkx in the establishment of a pro-inflammatory response, and (3) characterization of eosinophils in acute and chronic muscle damage. Mice deficient for Mkx exhibited delayed muscle regeneration, accompanied by impaired clearance of necrotic fibers and smaller regenerated fibers. This diminished regenerative capacity was associated with a reduction in the recruitment of pro-inflammatory macrophages to the site of damage. In culture, $Mkx^{-/-}$ bone marrow-derived macrophages displayed reduced proliferative capacity but retained the ability to polarize in response to a pro-inflammatory stimulus. The necessity of Mkx in mounting a robust immune response was further confirmed by an immunological challenge in which $Mkx^{-/-}$ mice exhibited increased susceptibility to infection by *Salmonella enterica* serovar Typhimurium. Significant downregulation of key cytokine and chemokine expression was identified throughout the course of muscle

repair in $Mkx^{-/-}$ mice and represents one mechanism in which Mkx regulates the establishment of an inflammatory response. Previous research discovered that Mkx is highly expressed in eosinophils, a type of innate immune cell that participates in disease-fighting and inflammation, however the role of eosinophils in muscle repair is not well described. This work outlines the contribution of eosinophils in muscle repair following acute and chronic injury. In healthy mice, eosinophils were found to inhibit efficient muscle repair following acute injury. Utilizing the $mdx^{-/-}utrn^{-/-}$ muscular dystrophy mouse model, eosinophil depletion via administration of anti-IL-5 antibody significantly improved diaphragm fiber diameter and increased the survival rate during the course of treatment.

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CHAPTER 1

INTRODUCTION

Tissue repair is an essential process that occurs throughout the body and over the course of every individual's lifetime. Disruption of tissue homeostasis results from numerous sources, including injuries, infections, diseases, and exposure to toxins. Following disruption of homoeostasis, an immediate inflammatory response is triggered by the release of damage- and pathogen-associated molecular patterns (DAMPs and PAMPs) from dead and dying cells and pathogens, respectively (Wynn & Vannella, 2016). Together, the innate immune response and cells of the tissue microenvironment coordinate in a complex and highly regulated manner to orchestrate tissue repair and the subsequent return to homoeostasis. Dysregulation of the repair process leads to chronic inflammation, accumulation of fibrotic tissue, and ultimately results in attenuated repair and function. Thus, regulation of the inflammatory response is critical for efficient repair and represents a therapeutic target to enhance tissue repair. In this work, skeletal muscle is used as a model system to study immune cell-mediated repair in the context of both acute and chronic damage.

To facilitate efficient muscle repair and develop therapeutics for large-scale muscle injuries and muscular dystrophies, an understanding of how the innate immune response coordinates with damaged myofibers is key. Several integral components, reviewed in greater detail in chapter 2, to this process have been thoroughly investigated over the years, however much is still unknown about the underlying mechanisms in which infiltration and function of extravasated innate immune cells within skeletal

muscle are regulated. Specifically, there is limited knowledge on the precise function of transcription factors expressed in cells of the myeloid and myogenic lineages during muscle repair.

In this work, the role of the transcription factor *Mohawk* (*Mkx*) in skeletal muscle repair is examined. *Mkx* is the only known member of its class within the superclass of the three-amino-acid-loop-extension (TALE) atypical homeobox genes, which are characterized by an additional three amino acids in loop 1 of the homeodomain (Ades & Sauer, 1995; Gehring et al., 1994; Noyes et al., 2008; Wolberger, 1996). Originally discovered in *Drosophila melanogaster*, the homeobox motif is highly conserved across animals, fungi, plants, as well as several single cell eukaryotes and functions in the regulation of pattern formation during development (Burglin & Affolter, 2016). The triple-alpha-helix binding domain of *Mkx* consists of a 180-base pair (bp) region, encoding for 60 amino acids, known as the homeodomain. The third alpha helix functions as the recognition helix and directly interacts with nucleotides in the major groove, while the N-terminal arm interacts with nucleotides in the minor groove. Specific amino acids in the N-terminal arm (namely 3, and 5-8) and the recognition helix (amino acids 47, 50, 51, 54, and 55) mediate DNA recognition through formation of the recognition motif TTACA (Ades & Sauer, 1995; Gehring et al., 1994; Noyes et al., 2008; Wolberger, 1996).

Mkx is among four other classes of homeobox genes (*Pbc*, *Meis*, *Iro*, and *Tgif*) within the TALE superclass and is most closely related to the Iroquois (Iro) family, differentiated by the presence of a highly-conserved IRO Box in the carboxy-terminal

half of *Irx* genes (Bürglin, 1997). The *Irx* genes regulate early embryonic patterning and specification, where they are especially important in the formation and development of the heart, nervous system, kidneys, lungs, pancreas, retina, female gonads, and limbs (K. H. Kim et al., 2012).

Utilizing whole-mount in situ hybridization (WISH) with an *Mkx*-specific probe, Anderson et al. characterized the expression of *Mkx* within the developing mouse embryo (2006). The first evidence of *Mkx* transcription was found in the anterior-most somites at E9.0, specifically within the dorsal region of dermomyotome. As development progressed (E9.0 - E11.5), expression migrated towards the somites located more posteriorly and was restricted to the epithelial dorsomedial lip (DML) and ventrolateral lip (VLL) of the dermomyotome. These regions consist of highly migrative and proliferative stem cells of the myogenic lineage, which give rise to skeletal muscle. At E10.5 expression expanded to prechondrogenic cells of the frontonasal mass, a region that eventually becomes the forehead, nasal cartilage, and philtrum. *Mkx* expression then shifted at E12.5 to the syndetome compartment, eventually giving rise to tendon, within the somite. Additionally, *Mkx* expression was detected in the ureteric buds of the kidney, the testis cords of the male gonad (Anderson et al., 2006), the otic vesicle, and the developing palate (Han Liu et al., 2006).

Postnatally, *Mkx* is perhaps best characterized in the tendon where it's expression is essential for normal morphogenesis and function (Huanhuan Liu, Zhu, et al., 2014). Upregulation of *Mkx* occurs during tendon maturation, following upregulation of the transcription factor *Scleraxis* (*Scx*) during tenocyte differentiation (W. Liu et al., 2010;

Murchison et al., 2007). When complexed with Smad2/3, MKX has been shown to function as a transcriptional activator promoting the expression of type 1 collagen (*Col1a1* and *Col1a2*), *Tenomodulin* (*Tnmd*), *Decorin* (*Dcn*), and *Transforming growth factor-β* (*Tgfβ*) in mesenchymal stem cells (MSCs) (Berthet et al., 2013; Huanhuan Liu, Zhang, et al., 2014). Mice lacking MKX exhibit a wavy-tail phenotype with hypoplastic tendons that are characterized by smaller tendon fibrils, reduced tensile strength, abnormal sheath development, and decreased levels of type I collagen (Col1a1 and Col1a2), fibromodulin, tenomodulin, and decorin (Ito et al., 2010; W. Liu et al., 2010).

Tendons, which connect muscle to bone, are load-bearing structures specially designed to withstand and transmit mechanical force. Interestingly, *Mkx* has been demonstrated to facilitate mechano-transduction in tenocytes by promoting the expression of downstream mechanical stress-activated extracellular matrix (ECM) genes (Kayama et al., 2016). Kayama and colleagues found that application of mechanical stimulation, via treadmill exercise, resulted in a failure of $Mkx^{-/-}$ tendons to respond to mechanical force, as demonstrated by a lack of increase in collagen fiber diameter and density (2016). While the regulation of *Mkx* activity is not well described, recent research has reported the induction of *Mkx* expression by general transcription factor II-I repeat domain-containing protein 1 (GTF2IRD1) following mechanical force (Kayama et al., 2016). In addition, the activation of *Mkx* and downstream ECM gene expression was dependent upon the load of mechanical force. Exactly how *Mkx* and GTF2IRD1 coordinate in response to varying loads remains unclear, however, these studies suggest

that *Mkx* functions as mechanosensor, essential for orchestrating remodeling of the tendon, by stimulating the expression of collagens and proteoglycans.

Although *Mkx*$^{-/-}$ mice do not display any skeletal muscle, bone, or cartilage defects, MKX has been shown to strongly repress the myogenic, osteogenic, adipogenic, and chondrogenic lineages (Anderson et al., 2009; Nakahara et al., 2013; Suzuki et al., 2016). Functioning as a transcriptional repressor, MKX was found to inhibit the myogenic conversion of 10T1/2 fibroblasts via recruitment of the Sin3A/HDAC complex (Anderson et al., 2009). Further, in the immortalized mouse myoblast cell line C2C12s, MKX was demonstrated to bind to the *MyoD* promoter thereby repressing its transcription and inhibiting subsequent myoblast differentiation (Chuang et al., 2014). In mouse satellite cells, *Mkx* was shown to promote specification of the slow fiber type, via repression of *Sox6* expression, during skeletal muscle differentiation (Anderson et al., 2012).

Recently, high expression of *Mkx* was demonstrated in eosinophils, a type of phagocytic white blood cell (de Graaf et al., 2016) normally involved in the allergic response and parasitic infection. The authors performed a database search to find genes with similar expression profiles to the well-characterized eosinophil receptor, CCR3. Among their results were known eosinophil associated genes, such as *Il4*, *Siglecf*, and *Epx,* as well as *Mkx,* which was previously not associated with eosinophils. The authors went on to describe *Mkx* as an eosinophil-specific transcription factor, demonstrating the greatest population of *Mkx*-positive cells in eosinophils derived from the peritoneal cavity. While eosinophils are known to infiltrate muscle during the pro-inflammatory

response following injury, little is known about their regulation or their precise function in muscle repair.

The established role of *Mkx* in repression of the myogenic lineage, along with its expression profile, brings about intriguing questions regarding *Mkx* regulation of muscle repair. In this work, the role of the transcription factor *Mkx* in orchestrating the pro-inflammatory response was identified (chapter 3). Further, because of high *Mkx* expression in eosinophils (de Graaf et al., 2016), the contribution of eosinophils in muscle repair following acute and chronic damage was investigated (chapter 4).

CHAPTER 2

DEPENDENCY ON NON-MYOGENIC CELLS FOR REGENERATION OF SKELETAL MUSCLE

Introduction

In the search to uncover the mechanisms of tissue regeneration and how they can be leveraged for therapeutic approaches, skeletal muscle has become an attractive model. Studies in the genetically tractable mouse have provided insight into the myogenic progenitor cells and signaling networks essential for efficient muscle repair in response to acute and chronic damage. More recently, it has become clear that crosstalk between muscle, the innate immune response and interstitial fibroblastic cells is essential for muscle regeneration. An imbalance in signaling, as observed with chronic inflammation of Duchenne's muscular dystrophy patients, can lead to a progressive increase in fibrosis, fat deposition and muscle necrosis. In contrast, de novo muscle regeneration in response to amputation or severe trauma is largely limited to amphibians, reptiles, and fish among the vertebrates. The additional layers of regulation are necessary to recruit progenitor cells to the site of the amputation as well as impose the positional identity required to accurately regenerate individual muscle groups. Similarly, myeloid and fibroblastic cells have also been shown to participate in these processes. In this chapter, we will review the recent advances in our understanding of the role of non-myogenic cells in muscle regeneration.

Satellite Cells of the Myogenic Lineage

Skeletal muscle regeneration is dependent on satellite cells that are functionally defined by their ability to both self-renew and differentiate into myoblasts that are able to fuse to form myofibers. These cells are maintained in a quiescent (G_0 phase) state until environmental cues associated with muscle injury stimulate re-entry into the cell cycle. During effective muscle repair, activated satellite cells migrate to the site of injury, proliferate, and differentiate to generate new muscle fibers.

Satellite cells are characterized by their location beneath the basal lamina of muscle fibers and constitutively express the transcription factors *Pax7* and *Myf5* (Cornelison & Wold, 1997; Seale et al., 2000). Ablation of *Pax7* results in decreased satellite cell proliferation and self-renewal, significantly impacting muscle growth and repair (Seale et al., 2000). Quiescent satellite cells (QSCs) have been found to express 500 genes not present in activated satellite cells that participate in cell–cell adhesion, negative regulation of the cell cycle, transcriptional control, and lipid and extracellular matrix transporter activity (Shea et al., 2010). Gene loci in QSCs that are only expressed at very low levels until induction via the onset of satellite cell activation are marked by histone H3 Lys4, a marker of active chromatin, indicating that these regions are open, awaiting the signals necessary to prompt activation and begin repair, and not in a dormant state (Fukada et al., 2007; Guenther et al., 2007). The ability of QSCs to immediately respond to injury stimuli allows for effective muscle repair.

Upon muscle injury, the myofiber sarcolemma and basal lamina are dismantled, resulting in a disconnection between satellite cells and the collagen-laminin network on

which they are anchored. This disruption of the myofiber allows for the release and entry of factors critical for satellite cell activation. One of the first factors implicated in activation, hepatocyte growth factor (HGF), is released from the basal lamina, it then proceeds to bind to the Met receptor on the surface of satellite cells, causing their activation and aiding in their migration to the injury site (Tatsumi et al., 1998). Dying fibers within the niche generate nitric oxide (NO), further stimulating HGF release from the basal lamina. Also implicated in the activation and proliferation of satellite cells is the Notch signaling pathway; blockage of Notch leads to inhibition of satellite cell proliferation, whereas up-regulation of Notch leads to the promotion of muscle regeneration (I. M. Conboy et al., 2003; Kuang et al., 2007). In the muscle niche itself, several factors are secreted that aid in multiple aspects of muscle repair. Fibroblast growth factor (FGF) secretion into the ECM activates the MAPK cascade, resulting in the activation and regulation of satellite cell quiescence (Jones et al., 2005). Phosphorylated p38 and MyoD are among the earliest markers of activation, with p38α/β MAPK inducing MyoD protein expression. In support of satellite cell proliferation, Notch3 mRNA and protein levels decline upon activation (Mourikis et al., 2012). Additionally, production of the MYF5 protein begins due to a decrease in miR-31 levels, giving activated satellite cells a *Pax7*$^{+}$, *Myf5*$^{+}$ phenotype.

Recently, an additional phase of satellite cell quiescence, termed the G_{alert} phase, has been identified in response to injury. Experiments performed by Rodgers et al. (2014), demonstrated that satellite cells residing in muscle in the leg contralateral to the limb with the induced injury were distinct from both quiescent and activated satellite

cells. In culture, QSCs in the G_{alert} phase were found to enter the cell cycle earlier than non-injury-induced QSCs. Additionally, G_{alert} phase QSCs demonstrated an increase in cell size as compared to QSCs, and a high transcriptional correlation between G_{alert} phase QSCs and activated satellite cells was identified. Both mTORC1 activity and HGF signaling were required for QSCs to switch from G_0 to the G_{alert} phase in response to injury. These findings suggest that G alert phase QSCs retain properties of both QSCs and activated satellite cells in a phase that is "primed" for injury response. In fact, QSCs of the G_{alert} phase demonstrated heightened differentiation in culture and enhanced regeneration following an induced injury in vivo (Rodgers et al., 2014).

Proliferation of satellite cell and myoblasts.

Satellite cell activation is followed by the rapid expansion of *Pax7*$^+$, *Myf5*$^+$ cells that will form the myoblast population, eventually participating in muscle repair, and self-renewal of a smaller population of *Pax7*$^+$, *Myf5*$^-$ satellite cells that will become quiescent in anticipation of later injury events (Figure 1). The majority of *Pax7*$^+$, *Myf5*$^+$ satellite cells undergo symmetric division, producing two *Pax7*$^+$, *Myf5*$^+$ progenitor cells. WNT7a, acting through its receptors FZD7 and VANGL2, induces symmetric cell division through the planar cell polarity pathway (Le Grand et al., 2009). In addition to HGF, insulin-like growth factor-1 (IGF-1), epidermal growth factor (EGF), transforming growth factors α/β (TGFα and TGFβ), and platelet-derived growth factor (PDGF) also contribute to the proliferation and differentiation of myoblasts (Wozniak & Anderson, 2007). Due to damage of the sarcolemma and basal lamina, myofibers receive an inflow of calcium from the (ECM) matrix, which aids in proteolysis of the myofiber (Oberc &

Engel, 1977). *Pax7*$^{+}$, *Myf5*$^{+}$ cells, stimulated through activated leukocyte secretion of IGF-1 and delivered through capillaries into the niche, will continue to proliferate through the down-regulation of $P27^{kip1}$ and through inactivation of the transcription factor FOXO1 (Chakravarthy et al., 2001). Negative mitogenic modulation of satellite cells exists through the transforming growth factor β (TGFβ) superfamily, most notably myostatin, which inhibit differentiation of satellite cells through down-regulation of MyoD expression and inhibits activation through the up-regulation of P21 and decreased levels of CDK2 (Langley et al., 2002; McCroskery et al., 2003). Tumor necrosis factor α (TNFα) also negatively mediates differentiation through the utilization of the TGFβ activated kinase (TAK1)/p38/ NF-kB pathway, resulting in increased levels of Activin A expression to support proliferation (Trendelenburg et al., 2012).

Approximately 10 % of the satellite cell population maintains a *Pax7*$^{+}$, *Myf5*$^{-}$ profile and will undergo asymmetrical division to give rise to one *Pax7*$^{+}$, *Myf5*$^{-}$ and one *Pax7*$^{+}$, *Myf5*$^{+}$ cell (Figure 1). Several signaling pathways present in the microenvironment of the satellite cell niche are responsible for controlling asymmetric satellite cell polarity and fate. Components of the Notch pathway, including a Notch3 effector protein, Notch ligand Delta1 (Dll1), and Notch agonist Numb have all been found to asymmetrically distribute between daughter cells, with DLL1 and NUMB found selectively in the daughter cell committed to becoming a myoblast (Irina M. Conboy & Rando, 2002; Kuang et al., 2007). Ablation of *Numb* in the muscle lineage profoundly decreased satellite cell proliferation, negatively affecting the ability of muscle to repair following an induced injury (George et al., 2013). Additionally, factors involved in cell

polarity determination, namely parts of the Par complex and Scribbled planar cell polarity protein (Scrib), have been implicated in asymmetric division. Orientation to the myofiber plays an important role in the ability of the satellite cells to asymmetrically divide. This relation to the myofiber, conferred by an apical-basal polarity, is dependent on the interaction of cell membrane receptors basal integrin α7β1 and apical M-cadherin, resulting in the production of one basal *Pax7*$^{+}$ *Myf5*$^{-}$ cell and one apical *Pax7*$^{+}$ *Myf5*$^{+}$ daughter cell (Kuang et al., 2007). It has also been proposed that the position of the mitotic spindle in relation to the myofiber axis plays a role in asymmetric division cell fate (Kuang et al., 2008).

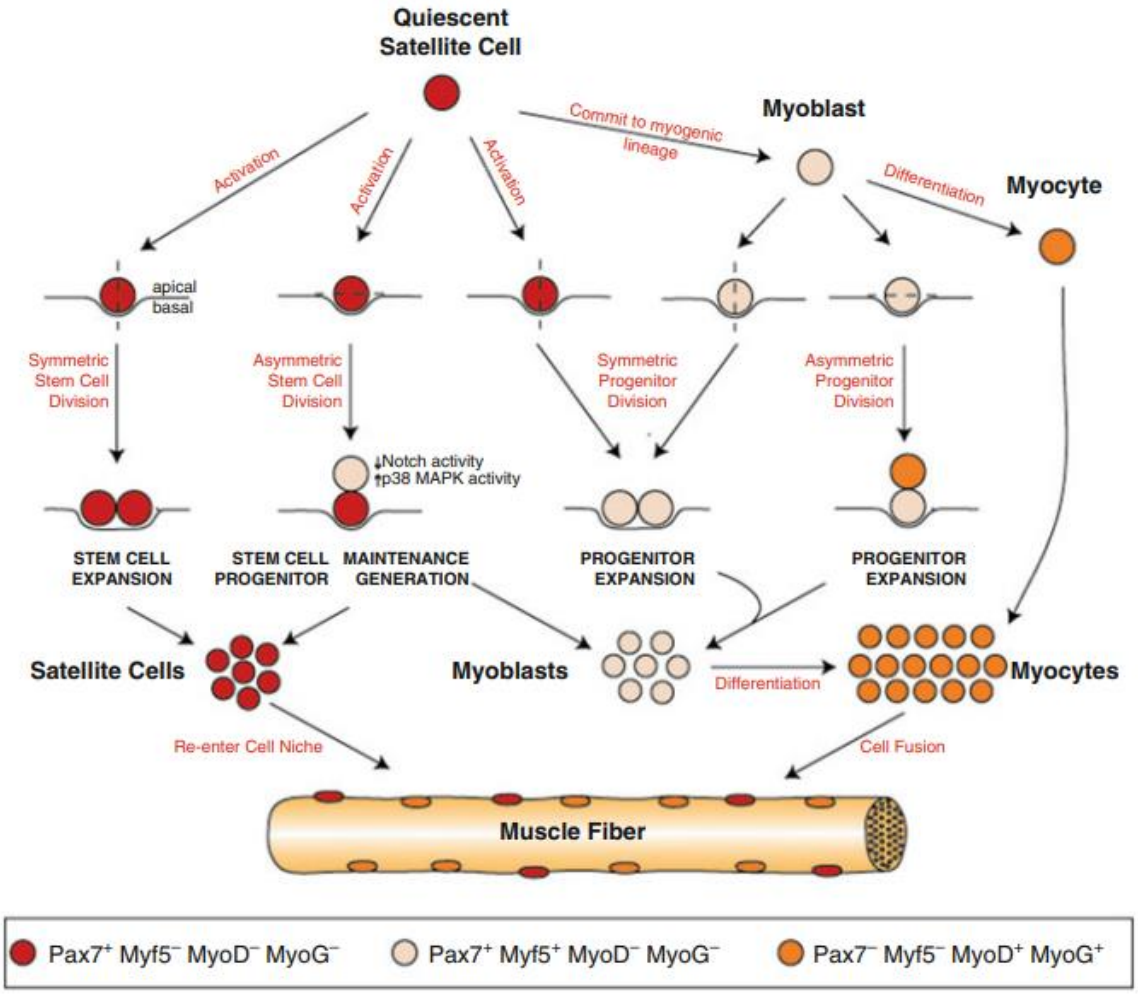

Figure 1. Mechanisms of satellite cell division for muscle maintenance and repair. Following entry to the cell cycle, quiescent satellite cells symmetrically or asymmetrically divide along the apical basal axis. Symmetric and asymmetric divisions lead to the generation of additional muscle stem cells and progenitor cells. Additionally, satellite cells can directly commit to the myogenic lineage and expand the progenitor cell population or differentiate into myocytes. Resulting muscle stem cells return to the niche to replenish the pool of quiescent satellite cells. Resulting myocytes fuse to form myotubes, leading to the formation of new muscle fibers.

Heterogeneity of the satellite cell population.

Studies in culture first revealed heterogeneity in the satellite cell population with a "responsive population" that readily proliferates in response to damage and participates in repair, and a "reserve population" that divides at a slow rate and is refractory to

differentiation into mature myotubes. This heterogeneity has been reported in muscle tissue at a ratio of 5:1 (responsive: reserve), confirming their relevance to normal muscle biology. The slow dividing cells contribute solely to skeletal muscle when transplanted back into mouse EDL muscle, confirming their commitment to the myogenic lineage. Genome-wide gene expression studies revealed differential expression between the two populations with reserve cells expressing higher levels of inhibitor of differentiation (Id) and other genes that confer "stemness". This predicts that the slow dividing cells that are refractory to repair signals, are essential to muscle homeostasis for long-term maintenance of the satellite cells population.

Satellite Cell Regulation Through the Stem-Cell Niche

The activation, migration, and proliferation of satellite cells are supported by the inflammatory microenvironment created by components of the niche and immune cells. In addition to ECM, the niche includes fibro-adipogenic (FAP) cells, vasculature, and both residential and infiltrating immune cells that are capable of direct communication with satellite cells. Oxygen free radicals released by neutrophils further break down the sarcolemma, while matrix metalloproteinases released by both damaged myofibers (MMP2) and immune cells (MMP9), aid in the degradation of ECM proteins (Kherif et al., 1999). ECM digestion through MMPs plays a vital role in satellite cell migration to the site of injury, especially in fibrotic tissue.

FAPs are bipotent fiber-associated cells that also proliferate in response to muscle fiber injury (Joe et al., 2010). FAPs double in number in less than 48 h and up-regulate the expression of Interleukin 6 (IL-6) roughly tenfold. IL-6, along with Wnt and IGFs,

has been implicated as a pro-differentiation signal that is essential for the differentiation and maturation of myoblasts during muscle repair (Joe et al., 2010; Otto et al., 2008; Serrano et al., 2008). During myolysis, FAPs have been found to assist in the clearing of cellular debris through phagocytosis of necrotic thymocytes, and when compared to macrophages, FAPs have been found to be fourfold more efficient in debris clearance (Heredia et al., 2013).

Microvasculature and accompanying pericytes help to sustain the cells of the microenvironment, as well as provide the necessary access to circulation for immune cell infiltration in response to damage and delivery of key factors that assist with niche maintenance and satellite cell regulation (Fukada et al., 2007; Machida & Booth, 2004; McCroskery et al., 2003). PDGF and vascular-endothelial growth factor (VEGF) are released from ruptured blood vessels in response to injury and play an important role in reciprocal communication with satellite cells to promote their proliferation, as well as angiogenesis (Montarras et al., 2013). Satellite cells are commonly found surrounding the vasculature within a 5μm radius, with up to 82% in murine models and 68% in human residing near capillaries (Christov et al., 2007). Pericytes in the muscle serve a jack of all trades role; they help to replace and regenerate the vasculature that can be lost or damaged due to muscle injury, also have been found to replace muscle, and become myogenic in vitro (Dellavalle et al., 2007). Pericytes have also been shown to give rise to most of the collagen forming cells during muscle injury, and, in the presence of neurons, have been shown to produce collagens I and III (Dulauroy et al., 2012).

The ECM contributes to the regulation of satellite cells in the niche. Proteoglycans and glycoproteins play a role in niche homeostasis and in the repair process. Collagen VI ablation in mice leads to a muscle wasting disease not dissimilar to the common dystrophic models (Bönnemann, 2011). ECM proteins bind to the transmembrane protein dystrophin, forming an anchor that connects the satellite cells to the basal lamina and maintains their anatomical location (Michele & Campbell, 2003). ECM proteins can also act as mitogens for satellite cells. Resting, non-damaged satellite cells are located in fibronectin rich regions of the myofiber niche, Syndecan4 (SYN4) and Frizzled7 (FZD7) on the satellite cells act as co-receptors to bind fibronectin (Bentzinger et al., 2013). In the presence of WNT7a, this complex will induce symmetrical division. Upon muscle damage, fibronectin is transiently expressed to help maintain the satellite cell pool through the Wnt signaling pathway (Bentzinger et al., 2013; Han et al., 2016).

The elasticity of the myofiber also plays a role in regulation; normal muscle fibers have a Young's modulus of approximately 12 kPa, while those in aged or dystrophic muscle are much stiffer (Engler et al., 2004; Gao et al., 2008). This leads to a decrease in quiescent satellite cells because the increased stiffness induces them to enter the cell cycle. Recent work using collagen based scaffolds with elasticity from 2 to 25 kPa as determined by atomic force microscopy (AFM), has shown that on substrates that measure 2 kPa most of the satellite cells maintain their quiescent states and do not enter the cell cycle. Whereas at 25 kPa only about 45 % remain quiescent in vitro (Quarta et al., 2016). These findings could explain why in aged or dystrophic muscle there is a

decreased satellite cell presence, as these two niche environments have an increased stiffness (Boontheekul et al., 2007; Quarta et al., 2016).

Innate Immune Response During Skeletal Muscle Repair

Regeneration of skeletal muscle cannot be accomplished solely by satellite cells. Several types of immune cells, both resident and infiltrating, play an indispensable role in effective tissue regeneration. In healthy homeostatic muscle, immune cells are kept at a minimum, however, disruption of the basal lamina and sarcolemma of myofibers initiates several waves of immune cell infiltration that play discrete roles in the removal of necrotic fibers, activation of satellite cells, and ultimately the efficient differentiation into mature muscle fibers. The majority of the immune cells involved in muscle repair are those of the innate leukocyte lineage—macrophages, neutrophils, dendritic cells, mast cells, eosinophils, basophils, and natural killer cells. Central to the innate immune response is the production and responsiveness to cytokines, chemokines, and growth factors. These signaling molecules mediate crosstalk with satellite cells and FAP cells during the repair process.

Immediately upon myofiber damage , resident mast cells within the muscle degranulate, releasing TNFα, while resident macrophages release C-C motif chemokine ligand 2 (CCL2) and C-X-C motif chemokine ligand 3 (CXCL3), recruiting transient polymorphonuclear neutrophils from the circulation to the site of injury (Brigitte et al., 2010). Satellite cells also contribute to chemoattraction to the site of damage through the release of the pro-inflammatory cytokines IL-1, IL-6, and TNFα (Tews & Goebel, 1996). Neutrophils rapidly invade the injured tissue in significant numbers and persist in the

tissue for approximately 24h, where they promote sarcolemma damage through the release of oxygen-free radicals (Tidball, 1995). Through the secretion of IL-1 and IL-8, neutrophils promote the recruitment of circulating $CX3CR1^{low}$, $Ly6C^{+}$, $CCR2^{+}$ phenotype monocytes to the site of injury (Saini et al., 2016) and binding of CCL2 and CCL7, by the C-C motif chemokine receptor, CCR2 (Tsou et al., 2007). Disruption of either receptor or ligands leads to severe deficits in monocyte recruitment and efficient muscle repair (Lu et al., 2011; Warren et al., 2005; Warren et al., 2004). The infiltrating monocytes differentiate into macrophage subtypes, both pro and anti-inflammatory, in a process that is highly dependent on the tissue microenvironment. At approximately 24-h post muscle injury, monocytes/macrophages begin to express high levels of IL-6, supporting macrophage infiltration and myoblast proliferation through the STAT3 pathway. Effective muscle repair requires sufficient generation of myoblasts for regeneration of the damaged tissue. Knockout of IL-6, or knockdown of STAT3, resulted in decreased MyoD, Myogenin, and macrophage infiltration, ultimately resulting in diminished muscle repair (Zhang et al., 2013).

Initially, the pro-inflammatory phenotype is maintained as neutrophils secrete Th1 inflammatory cytokines, interferon-gamma (IFNγ) and TNFα, to induce monocytes to polarize into M1 macrophages ($CX3CR1^{low}$, $Ly6C^{+}$, $CCR2^{+}$). In addition to IFNγ and TNFα, pathogens and granulocyte macrophage colony-stimulating factor (GM-CSF) are capable of stimulating M1 macrophage polarization (F. O. Martinez & Gordon, 2014) (Figure 2). M1 macrophages phagocytose cellular debris and secrete factors, such as IL-1b and IL-12, to recruit additional inflammatory cells for debris clearance and pathogen

removal. Nitric oxide (NO), produced by M1 cells acts to lyse cells for removal, however, if dysregulated, it can lead to increased tissue damage (Nguyen & Tidball, 2002). During the pro-inflammatory phase, which occurs approximately 24–96 h post injury, the NF- kB pathway in both macrophages and myoblasts is activated in response to TNFα. In macrophages, this enhances the inflammatory response by stimulating the release additional pro-inflammatory cytokines. In muscle, CyclinD1 expression is induced, while MyoD expression is suppressed, in response to activation of the NF-kB pathway, supporting myoblast proliferation and preventing differentiation (S.-E. Chen et al., 2005; Langen et al., 2004).

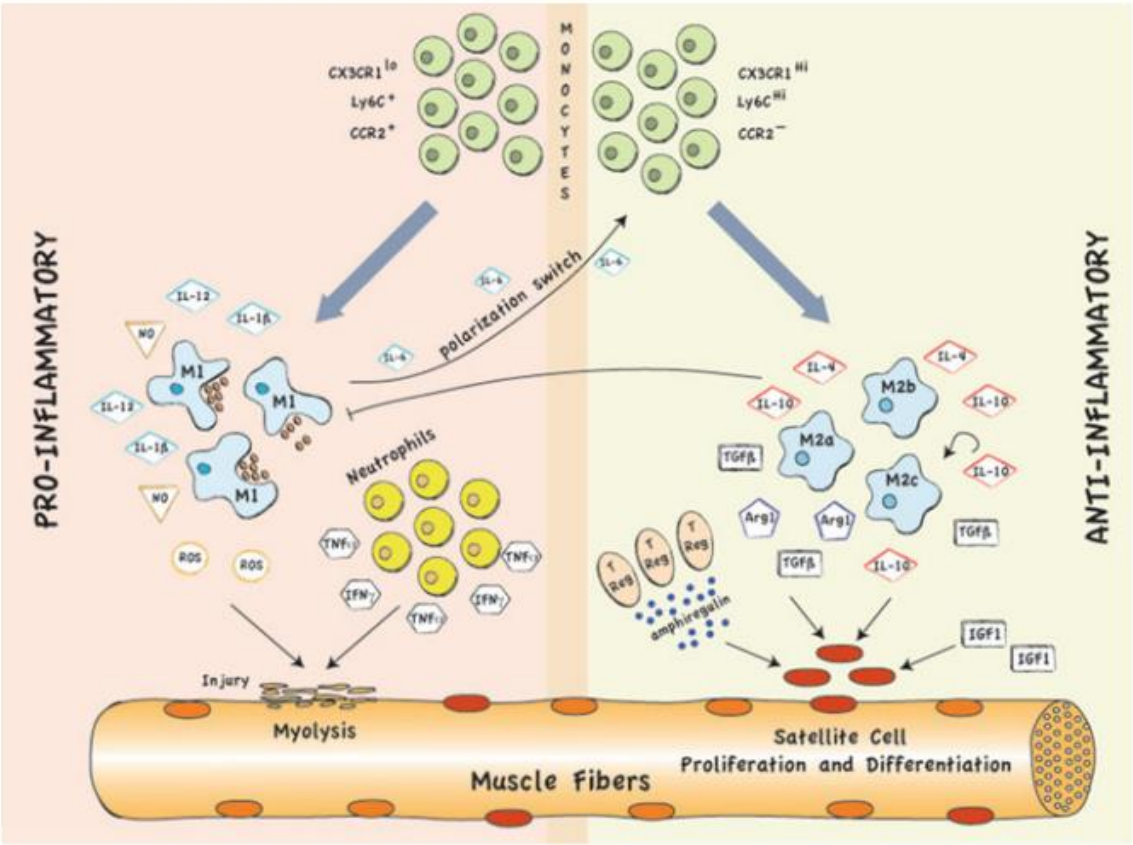

Figure 2. Immune cell contribution and modulation in damaged muscle tissue. In response to myofiber injury, neutrophils from circulation invade the site of damage where they aid in further tissue break down and recruit CX3CR1^{Lo}, Ly6C^{+}, CCR2^{+} monocytes, differentiating into M1 macrophages, for continued debris clearance and pro-inflammatory cytokine secretion. M1 phagocytosis induces macrophage polarization towards an anti-inflammatory phenotype to support muscle repair. CX3CR1^{Hi}, Ly6C^{Lo}, CCR2^{-} monocytes differentiate into M2a, M2b, and M2c macrophages, functioning to suppress inflammation and promote satellite cell proliferation and differentiation. T regulatory cells assist M2 macrophages in resolving inflammation and fostering muscle repair.

Phagocytosis by M1 macrophages and exposure to CSF-1 induce macrophage polarization to skew from a pro-inflammatory phenotype towards an anti-inflammatory phenotype, resolving the inflammation and beginning the muscle repair process (Arnold et al., 2007). Infiltrating monocytes now become CX3CR1^{hi}, Ly6C^{-}, CCR2^{-} and differentiate into three subtypes of M2 macrophages. Several molecules have been

identified as regulators of the switch from early pro-inflammatory to late anti-inflammatory macrophage phenotypes. cAMP response element-binding protein (CREB), a multifunctional transcription factor, is critical for the up-regulation of genes associated with M2 macrophages (IL-10, IL-13R, Arg-1) and repression of M1 macrophage activation (Ruffell et al., 2009). Mitogen-activated protein kinase (MAPK) phosphatase-1, through inhibition of p38 MAPK activation, functions to control macrophage subtype shifting. MAPK also helps to resolve inflammation to allow for proper muscle repair (Perdiguero et al., 2011). Recently, AMP-activated protein kinase (AMPK), widely known as a regulator of metabolic homeostasis, has also been identified as a regulator of macrophage polarization skewing. Mounier et al. (2013), demonstrated loss of M2 macrophage functionality and a loss of M2 markers expressed in AMPK$\alpha1^{-/-}$ macrophages. Further, AMPK$\alpha1^{-/-}$ mice showed deficient muscle repair resulting from a failure of M1 macrophage phagocytosis-induced polarization to an M2 phenotype (Mounier et al., 2013).

M2a macrophages arise from the release of IL-4 or IL-13 and signal via IL-4 receptor alpha (Novak & Koh, 2013). Release of these Th2 inflammatory cytokines causes increased expression of CD206 and CD36 by macrophages. In vitro, it has been shown that M2a macrophages, producing arginase, decrease M1 macrophage lysis activity through competition for arginine, the shared enzymatic substrate of arginase and iNOS (Villalta et al., 2008). M2a macrophages secrete IL-10 and TGF-β, thereby inducing the anti-inflammatory M2c macrophage subtype, which aids in IL-10 and TGF-β release (Figure 2). Secretion of these cytokines suppresses inflammation and promotes

satellite cell proliferation, allowing for remodeling of the extracellular matrix, angiogenesis, and muscle fiber development to begin (Villalta et al., 2008). Glucocorticoids and IFNβ can also stimulate the induction of the M2c subtype (Wermuth & Jimenez, 2015). The release of IL-4 by M2b regulatory macrophages, Th2 cells, eosinophils, and basophils further promotes the wound healing phase by decreasing phagocytosis and stimulating macrophage fusion (Wermuth & Jimenez, 2015). In addition to IL-4, the release of IGF-1 also contributes to continued satellite cell growth and myofiber fusion (Forbes & Rosenthal, 2014). In recent experiments by Tonkin et al. (Tonkin et al., 2015), macrophages were identified as a major contributing source of IGF-1 at the site of muscle damage. Indeed, when muscle injury is induced in mice devoid of IGF-1 in myeloid cells, a loss of regenerative capacity is demonstrated. During the late stages of healthy muscle repair, $Ly6C^+$ monocytes/macrophages and $CD206^+$ macrophages were found to express high levels of IGF-1. However, when IGF-1 is knocked out from myeloid cells, the population of $Ly6C^+$ monocytes/macrophages is heightened while the population of $CD206^+$ macrophages is diminished (Tonkin et al., 2015).

Aiding in the establishment of the anti-inflammatory environment at the site of muscle damage, a population of CD4 + regulatory T cells (T_{reg}) arises concurrently with M2 macrophages, though to a much lesser extent (Figure 2). FoxP3, a forkhead transcription factor, regulates T_{reg} cell lineage specification, however, it remains unclear whether the population of T_{reg} cells at the site of muscle injury derives from resident T_{reg} cells in the muscle or is recruited in response to damage. T_{reg} cells have been shown to

influence myeloid and T cell infiltration, as well as satellite cell colony-forming capacity. Additionally, T_{reg} cells were found express IL-10 and amphiregulin, which accumulate during the final stages of muscle repair and play important roles in negative regulation of inflammation and satellite cell activation and proliferation, respectively (Burzyn et al., 2013). Due to the capability of T_{reg} cells to modulate the inflammatory response and satellite cell activity, research in using T_{reg} cells to improve muscle repair is of current interest. Villalta et al. demonstrated increased levels of T_{reg} cells in both human Duchenne's muscular dystrophy (DMD) and in the corresponding mdx mouse. When T_{reg} cells are depleted from dystrophic muscle, a heightened Th1-cell-mediated response occurs causing increased myofiber damage (Villalta et al., 2014).

In recent years, the multi-faceted role of macrophages in wound repair has begun to lend itself to potential use in therapy for muscle injury. M1-polarized macrophages delivered to the site of muscle damage resulted in enhanced recovery of functionality with reduced myofiber damage and collagen accumulation (Rybalko et al., 2015). When M2a or M2c macrophages are injected, an increase in tube-like structures is observed, indicating improved angiogenesis (Jetten et al., 2014). To further aid in the repair of muscle injury, especially in cases of volumetric muscle loss, tissue scaffolds with inert or biodegradable properties have been the predominating focus. Contrary to avoiding an immune response, recent work has sought to take advantage of immune cells in the delivery of tissue scaffolds—now termed "smart scaffolds". Macrophages and other inflammatory cells, such as cytokines capable of modulating macrophage polarization, can be loaded into tissue scaffolds prior to transplantation, allowing for a therapeutic

approach that is personalized and works in conjunction with the patient's own immune response to enhance the repair process. Through an injectable multidomain peptide scaffold engineered by Kumar et al. the potential to recruit specific inflammatory cells and deliver cytokines to the site of injection was shown. MCP-1 and IL-4 loaded hydrogel scaffolds were capable of boosting macrophage recruitment and stimulating polarization towards a pro-healing M2 phenotype in a time-controlled manner, without inducing a local inflammatory response (V. A. Kumar et al., 2015).

De novo regeneration of skeletal muscle.

As described above, mammalian models have been powerful tools in parsing the signaling pathways regulating the regeneration of skeletal muscle in response to acutely damaged muscle. However, de novo muscle regeneration in response to amputation is largely limited to amphibians, reptiles and fish among the vertebrates. This process can be distinguished by the additional layers of regulation necessary to recruit progenitor cells to the site of the amputation and a complex set of temporal and spatial signals necessary to impose the positional identity required to accurately recapitulate individual muscle groups and coordinate the regeneration of distinct cell lineages that give rise to the skeletal elements, connective tissue, nerves, vasculature, and skin (Carlson, 2003). As with tissue repair, the study of skeletal muscle regeneration has been central to our understanding of complex tissue regeneration. Non-myogenic cell types have been implicated in this process. In this section, we will compare the regulation of muscle repair to regeneration through the lens of the microenvironment created by the immune cells and myofibroblasts.

Amphibians as a model for the study of skeletal muscle regeneration.

Members of the Anura (frogs and toads) and Caudata (salamanders and newts) orders are the most commonly studied amphibians for muscle regeneration. Anurans possess distinct developmental windows preceding metamorphosis where complete regeneration of organs can occur, while the urodeles (Caudata) are able to regenerate a wide variety of organs throughout adulthood. Perhaps the best studied regenerative tissue system has been limb and tail amputations that follow a conserved set of temporal events that include (1) a modified wound healing process , (2) progenitor cell recruitment and (3) activation and tissue rebuilding (reviewed in (Carlson, 2003; Kragl et al., 2009; Makanae & Satoh, 2012)). Conserved regulatory pathways shared between amphibian models has provided insight into how regeneration has been maintained in these animals and largely lost in mammals.

Wound healing and ECM remodeling during regeneration.

Wound healing associated with regeneration shares many common features with scar-free wound healing associated with skin repair. Within hours of amputation, epithelial cells and dermal fibroblasts migrate to the site of injury and cover the fi brin blood clot. The regenerative epithelial cells thicken to form an apical ectodermal cap (AEC) reminiscent of the apical ectodermal ridge (AER) that appears during limb development. The AEC promotes the remodeling of the basement membrane ECM through recruitment of leukocytes and the release growth factors that are capable of inducing the subjacent mesenchymal cells to form a blastema of undifferentiated proliferating progenitor cells with the ability to rise to the distinct cell types of the limb

(Christensen & Tassava, 2000; Christensen et al., 2002; J. Godwin et al., 2014). In the case of skeletal muscle, progenitor cells can be derived from myoblasts (*Pax7*$^{-}$, *MyoG*$^{+}$) that dedifferentiate muscle fibers and aid in the recruitment of satellite cells (*Pax7*$^{+}$, *MyoG*$^{-}$) (Morrison et al., 2006; Tanaka et al., 1997).

The ECM at the site of the wound is recognized as an important regulator of wound healing and the progression towards regeneration. ECM is a complex network of proteins composed primarily of collagens, laminins and fibronectins that interact to create scaffolding as well as serve as adhesion sites for cells through integrin binding. Small leucine-rich proteoglycans within the ECM bind growth factors and cytokines that create microenvironment niches for cell signaling (Sorokin, 2010). Within hours of amputation, migrating epithelial cells express matrix metalloproteinases (MMP) that promote ECM breakdown through the digestion of collagen. This facilitates cell invasion, debris clearance and release of the growth factors and cytokines that promote cell migration (J. Godwin et al., 2014; Konttinen et al., 2011). A second wave of MMP expression after 3 days is believed to participate in ECM remodeling and promoting muscle dedifferentiation (Nambiar et al., 2008). Treating newt wounds with MMP inhibitors resulted in shortened stumps with distal scars, indicating the importance of the ECM remodeling during regeneration (Vinarsky et al., 2005). Macrophages represent important regulators of ECM breakdown and remodeling at the wound site. Inflammatory cytokines produced by macrophages regulate ECM production from fibroblasts and myofibroblasts and ensure a pro-regenerative microenvironment at the site of the wound instead of an acellular fibrotic scar (Kovacs, 1991; Moyer & Wagner, 2011). Depletion of

macrophages in salamanders inhibits limb regeneration and promotes the formation of a distal scar and an overrepresentation of myofibroblasts (J. W. Godwin et al., 2013). This underscores the important relationship between the organism's ability to remodel ECM and the formation of fibrotic scars that prevent regeneration. In support of this, salamanders maintain the expression of other developmentally regulated collagens III and XII, tenascin, and hyaluronic acid later into adulthood than mice and delay the onset of collagen I that gives rise to acellular scars through cross-linking with heparin sulfate proteoglycans (J. W. Godwin et al., 2013; Seifert et al., 2012).

Myogenic progenitor cells during regeneration.

In classic experiments initially performed in salamanders, myogenic progenitor cells contributing to the blastema were found to be derived through the dedifferentiation of injured muscle (Echeverri et al., 2001; Hay, 1959; Lo et al., 1993). Dedifferentiation is characterized by a loss of differentiated muscle-specific markers, fragmentation of multinucleated myotubes into mononucleated cells and re-entry into the cell cycle (Duckmanton et al., 2005). The resultant mononucleated $Pax7^{-}$ $MyoG^{+}$ cells are capable of redifferentiation into muscle (Calve & Simon, 2011). Cre-loxP-based genetic fate mapping experiments have demonstrated that cells generated through dedifferentiation remain restricted to the myogenic lineage and are unable to contribute to other tissues of the limb or tail (Kragl et al., 2009; Sandoval-Guzmán et al., 2014).

Several transcription factors and cell cycle regulators have been shown to regulate muscle dedifferentiation (Echeverri & Tanaka, 2002; A. Kumar et al., 2004; Pajcini et al., 2010; Tanaka et al., 1997). Perhaps the best studied are members of the MSX family of

the homeodomain-containing transcription factors (MSX1 and MSX2) that have been implicated in maintaining cells in proliferative, progenitor state during limb development across vertebrates. Over expression of either MSX1 or MSX2 is sufficient to drive myotube dedifferentiation in culture and the formation of differentiation-competent myoblasts (A. Kumar et al., 2004). More recently, it was found that the LIM homeobox transcription factor, *Lhx2*, which can suppress muscle-specific transcription and differentiation in C2C12 cells, is a direct regulator of Msx1 and Msx2 transcription (Kodaka et al., 2015). Further, ectopic expression of MSX1 or MSX2 can induce dedifferentiation of mammalian myotubes suggesting the elements of the dedifferentiation regulatory network of the amphibians have been retained in mammals (Odelberg et al., 2000; Yang et al., 2014; Yilmaz et al., 2015).

Inactivation of the tumor suppressor Retinoblastoma (Rb) through phosphorylation has also been implicated in muscle regeneration in the newt limb, consistent with the requirement for reinitiating the cell cycle during generating progenitor cells (Tanaka et al., 1997). Inactivation of Rb is sufficient to promote DNA synthesis in differentiated mouse muscle in culture, however, the cells will not progress to proliferating myoblasts with the capacity for redifferentiation (Camarda et al., 2004; Sacco et al., 2003). Complete recapitulation of the dedifferentiation pathway requires an additional insult to the p53 signaling pathway through inactivation of the Alternate Reading Frame (ARF) of the *Ink4a* locus (Pajcini et al., 2010). Interestingly, the earliest identified *ARF* ancestor is in chickens, with no candidates in databases for non-amniote organisms (Brookes et al., 2004; Gilley & Fried, 2001; S. H. Kim et al., 2003). This

raises the possibility that loss of regenerative capacity in mammals is related to acquisition of additional levels of cell cycle regulation. There is evidence that environmental cues participate in the regulation of muscle fiber dedifferentiation. The ECM in the tissue proximal to the site of amputation undergoes a shift from a collagen and laminin based stiff ECM to a softer transitional ECM rich in hyaluronic acid, tenascin-C and fibronectin. Under cell culture conditions, this ECM differentially directs DNA synthesis, migration, myotube fragmentation and myoblast fusion (Calve et al., 2010; Calve & Simon, 2012).

In addition to the generation of *Pax7*$^{-}$, *Myog*$^{+}$ myoblasts through dedifferentiation, there is evidence that recruitment of *Pax7*$^{+}$, *Myog*$^{-}$ satellite cells from muscle proximal to the site of amputation participates in muscle regeneration in salamanders (Morrison et al., 2006). Further, cultured satellite cells are able to contribute to muscle regeneration upon transplantation (Kragl et al., 2009; Morrison et al., 2010). This indicates that the system for recruiting myogenic progenitor cells in mammals can participate in regeneration in amphibians as well. Cre-loxP-based genetic fate mapping approaches have been used to track cells in the blastema that are *Pax7*$^{-}$, *Myog*$^{+}$ and *Pax7*$^{+}$, *Myog*$^{-}$ (Kragl et al., 2009; Sandoval-Guzmán et al., 2014). Surprisingly, there was a preference for the recruitment of a premyogenic cell source between urodeles, with the *Notophthalmus viridescens* (newt) depending on dedifferentiation of muscle while the *Ambystoma mexicanum* (axolotl) leverages satellite cells (Sandoval-Guzmán et al., 2014). The newt employs a dedifferentiation strategy for the regeneration of other tissues, including the lens of the eye, while the axolotl has limited regenerative capacity for the

lens (Grogg et al., 2005; Suetsugu-Maki et al., 2012). This reveals a divergence in strategies for generating progenitor cells for tissue of two urodeles separated by approximately 100 million years. This raises interesting questions about the evolutionary pressures that would maintain two discrete mechanisms. The selection process has been strong enough that mammalian muscle is able to functionally recapitulate dedifferentiation with relatively small changes in gene expression of extracellular matrix.

Role of pro- and anti-inflammatory immune response in regeneration.

The duality of the innate immune response with the pro-inflammatory arm directed by Th1 cytokines and the anti-inflammatory arm directed by Th2 cytokines is conserved in urodeles. However, analysis of the cytokines post limb amputation reveals two overlapping spikes in Th1 and Th2 cytokines as well as CCL and CXCL chemokines at days 2 and 7, which predicts that anti-inflammatory M2 macrophages are recruited concurrently to the site of injury with pro-inflammatory M1 macrophages (J. W. Godwin et al., 2013). This is in contrast to mammalian muscle repair, where a distinct early wave of pro-inflammatory M1 macrophages is followed by anti-inflammatory M2 macrophages. The presence of M2 macrophages and Th2 cytokines did not inhibit the phagocytic activity of M1 macrophages in the first 24h post-amputation in the salamander, suggesting a different functional relationship between the two cell types during regeneration. Interestingly, M1 macrophage activity requires expression of anti-inflammatory cytokines as well as several signaling pathways critical for regeneration, including metalloproteinases MMP9 and MMP3, dedifferentiation regulator *Msx2*, blastemal markers *Prrx1* and *Sp9*, the production of Th2 cytokines, and TGFβ signaling

(J. W. Godwin et al., 2013). Thus, despite the temporal overlap, modulation of the pro-inflammatory immune response is essential for promoting regeneration.

Studies in Anurans, where regenerative capacity is limited to a pre-metamorphosis time period provides an opportunity to compare cellular processes associated with repair in permissive and non-permissive stages to examine mechanisms by which the immune system regulates regeneration (reviewed in (King et al., 2012)). *Xenopus*, the most common anuran model, will undergo complete limb or tail regeneration between pre-metamorphosis stages 50–53. After metamorphosis has started (stages 57–60), regeneration is only partially complete as exemplified by a cartilaginous spike replacing an amputated limb. The shift from tadpole to adult is associated with immunological shifts from a relatively simple "ancestral" system to one that is more complex and resembles that of the mammals (Flajnik et al., 1987; Robert & Cohen, 1998). Consistent with this, differential gene expression studies between regeneration competent and incompetent stages confirms differences in the immune signaling and resolution of inflammation (Grow et al., 2006; King et al., 2009; Pearl et al., 2008). While pro-inflammatory signals spike early after limb amputation in stage 53 of *Xenopus*, they persist at the regeneration non-competent stage 57 (King et al., 2009). This would indicate that unresolved inflammation in response to injury contributes to the loss of the regenerative capacity in adult frogs. In support of this, immune cell depletion can extend the period of regeneration competence in *Xenopus* (Fukazawa et al., 2009).

Studies in anurans and urodeles have provided seemingly conflicting models of the role of the inflammatory response to regeneration, with disruption of inflammatory

macrophages inhibiting salamander and newt regeneration while extending the regenerative refractory period in frogs (Fukazawa et al., 2009; J. W. Godwin et al., 2013). This can best be reconciled through the lens of comparative strength of the immune system. Salamanders are considered to have a strong innate immune system, but because of the lack of key adaptive immune responses, it is considered relatively weak compared to the frog and mouse (G. Chen & Robert, 2011). In the case of the frog, the strength of the immune system increases with age, leading to the hypothesis that the regenerative capacity of the organism is inversely proportional to the strength of the immune response to injury. This is likely an oversimplified axiom as phagocytotic macrophages are essential for salamander limb and tail regeneration. There has been considerable effort to understand the immune response to pathogens and this can provide insight into differences in humoral and cytotoxic immune response between amphibians (Cotter et al., 2008; J. Godwin et al., 2014; Kaufman et al., 1995). Understanding how the broader immune system plays a role in tissue regeneration should help resolve this confusion.

CHAPTER 3

THE TRANSCRIPTION FACTOR MOHAWK FACILITATES SKELETAL MUSCLE REPAIR VIA MODULATION OF THE INFLAMMATORY ENVIRONMENT

Abstract

Efficient repair of skeletal muscle relies upon the precise coordination of cells within the satellite cell niche and innate immune cells that are recruited to the site of injury. Pro- and anti-inflammatory cytokines and chemokines expressed by cells of the myogenic and myeloid lineages lead to highly integrated temporal and spatial control of muscle repair. Regulating the expression of these signals is believed to generate ideal targets to enhance skeletal muscle regeneration for individuals with volumetric muscle loss and chronic injuries associated with muscular dystrophy. The transcription factor Mohawk (*Mkx*) functions as a repressor of myogenic differentiation and plays a role in regulating fiber type specification. *Mkx* is expressed embryonically in all progenitor cells of the musculoskeletal system. In adults, *Mkx* is strongly expressed in hematopoietic stem cells that are integral in the inflammatory response. Mice deficient for MKX exhibit normal skeletal muscle development, but fail to repair muscle damage within the normal timeframe of 14-21 days. This deficiency in the repair process was accompanied by an imbalance in the temporal regulation of M1 and M2 macrophage populations. Additionally, over the course of muscle repair, genes critical in mediating the inflammatory response and subsequent muscle repair were significantly downregulated. *In vitro* culture of MKX-deficient bone marrow-derived macrophages (BMDM) revealed a delay in proliferation, while treatment with a pro-inflammatory stimulus indicated

proficient capacity of *Mkx*$^{-/-}$ BMDM to polarize to the M1 subtype. These studies demonstrate a critical role for *Mkx* in adult skeletal muscle repair that appears to be mediated through the initial activation of the inflammatory response.

Introduction

Skeletal muscle possesses an intrinsic ability to repair itself in response to traumatic or chronic injury. This is dependent on the crosstalk of signaling between the myogenic satellite cells and the innate immune system (Arnold et al., 2007; Tidball & Villalta, 2010). Damage to the myofiber leads to the release of chemokines and Th1 cytokines that both activate and expand a population of satellite cells, in addition to initiating a pro-inflammatory response that directs myolysis. This is followed by a transition to the release of Th2 cytokines that promote differentiation and fusion of satellite cells as well as activation of an anti-inflammatory response. Disruption of these signals promotes fibrotic scaring and reduced muscle function. Understanding the regulation Th1 and Th2 expression during skeletal muscle injury is essential for promoting robust tissue repair and mitigating fibrosis.

Damage to the myofiber initiates rapid recruitment of neutrophils, mediated by the release of chemoattractant CXC-chemokine ligand 1 (CXCL1) from resident macrophages. Release of interleukin-1 (IL-1) and interleukin-8 (IL-8) from neutrophils and CC-chemokine ligand 2 (CCL2) from resident macrophages recruit circulating monocytes (Fujishima et al., 1993; Lu et al., 2011). Monocytes differentiate into a pro-inflammatory subtype, termed M1 macrophages that are characterized by expression of F4/80, Ly6C, and CD11b (Brigitte et al., 2010). M1 macrophages clear necrotic fibers by

phagocytosis and promote satellite cell activation and proliferation through the release of reactive oxygen species and inflammatory cytokines, such as IFNγ and TNFα (Cheng et al., 2008; Collins & Grounds, 2001). M1 macrophages reach peak numbers 1-2 days following injury before progressively transitioning to an anti-inflammatory and pro-regenerative M2 macrophage phenotype. M2 macrophages, reaching peak numbers 3-5 days post-injury, express the mannose receptor CD206 and secrete cytokines (i.e. IL-10 and TGFβ) to aid in the resolution of inflammation and differentiation of satellite cells (Arnold et al., 2007; Villalta et al., 2011).

The timely initiation of inflammation and its subsequent cessation during muscle regeneration is critical for efficient repair. Reduced infiltration of M1 macrophages due to genetic ablation of *Ccl2* or its receptor *Ccr2* results leads to diminished growth of newly repaired fibers after injury (Lu et al., 2011; C. O. Martinez et al., 2010). Similarly, depletion of $F4/80^{+}$ macrophages at the time of transition from a pro-inflammatory to a pro-regenerative environment results in reduced satellite cell differentiation and impaired muscle regeneration (Tidball & Wehling-Henricks, 2007). Alternatively, chronic inflammation associated with muscular dystrophies leads to accumulation of fibrotic scarring and severe loss of function. Continuous expression of pro-inflammatory cytokines exacerbates muscle damage by halting the transition of M1 macrophages to the M2 subtype and inhibiting the apoptosis of fibro-adipogenic progenitors (FAPs). At the time of muscle injury, undifferentiated FAPs rapidly proliferate and aid in muscle repair by inducing the differentiation of activated myoblasts (Joe et al., 2010). However, under chronic inflammatory conditions aberrant macrophage secretion of TGFβ causes FAPs to

differentiate into fibroblasts, leading to increased deposition of ECM (Lemos et al., 2015; Uezumi et al., 2011). Understanding the regulation of the highly coordinated and intricate signaling network that exists between the satellite cell niche and the innate immune response is critical for the development of therapeutics, as much is still unknown about the signals that regulate efficient regeneration.

Mohawk (*Mkx*), a member of the TALE superclass of atypical homeobox genes, has been identified as a key regulator of both skeletal muscle and tendon differentiation. In muscle, *Mkx* functions as a repressor of myogenic differentiation (Anderson et al., 2009) and plays a role in the regulation of fiber type specificity during skeletal muscle differentiation, likely through a mechanism involving the downregulation of *Sox6* (Anderson et al., 2012). In tendon, the absence of MKX causes a significant reduction of extracellular matrix components collagen I, fibromodulin, and tenomodulin leading to hypoplastic tendons with decreased tendon size, growth, and abnormal sheath development (Ito et al., 2010; W. Liu et al., 2010; Suzuki et al., 2016). In tendon-derived cells (TDCs) lacking MKX, the differentiation of chondrogenic and osteogenic cells was markedly enhanced, while over-expression of *Mkx* lead to decreased differentiation of chondrogenic, osteogenic, and adipogenic cells (Suzuki et al., 2016). *Mkx* has also been identified as a key transcription factor in the development, maintenance, and regeneration of the annulus fibrosus (Nakamichi et al., 2016).

In the mouse, *Mkx* is expressed in several tissue types, including the progenitor cells of muscle, tendon, cartilage, and bone (Anderson et al., 2006), as well as in hematopoietic stem cells. Recently, *Mkx* was identified as an eosinophil-specific

transcription factor with an expression profile similar to C-C Motif Chemokine Receptor 3 (CCR3), which is involved in the infiltration and activation of eosinophils during inflammation (de Graaf et al., 2016). This expression pattern, along with the previous characterization of *Mkx* function in myogenic and tenogenic differentiation suggest that *Mkx* may play an important role in muscle repair. Evaluation of *Mkx*-deficient mice (*Mkx*$^{-/-}$) revealed a critical role for the gene in the temporal regulation of pro- and anti-inflammatory macrophage populations in response to muscle damage *in vivo*. Mice lacking MKX demonstrated delayed muscle regeneration and aberrant macrophage polarization, indicating a role for *Mkx* in skeletal muscle inflammation and repair.

Materials and Methods

Mice and genotyping. *Mkx*$^{-/-}$ mice on a 129-C57BL/6 mixed background have been previously reported (W. Liu et al., 2010). Wild-type (WT) C57BL/6J mice were purchased from The Jackson Laboratories (Bar Harbor, ME, USA). *Mkx*$^{-/-}$ and WT mice were bred and used in accordance with the Institutional Animal Care and Use Committee (IACUC) at Arizona State University (ASU). Mice were housed in sterile, pathogen-free isolation cages and monitored by the Department of Animal Care and Technologies (DACT) at ASU. *Mkx*$^{-/-}$ mice were genotyped and maintained as previously described (Anderson et al., 2012).

Muscle injury. Controlled injury of the right quadriceps of *Mkx*$^{-/-}$ mice and *Mkx*$^{+/+}$ age-matched control mice at 3 months of age was induced by injection of 50µl of cardiotoxin (10µM) (Latoxan). In some cases, 24-hours prior to tissue harvesting, an i.p. injection of 1% Evans blue dye (Sigma-Aldrich) was administered to allow for

visualization of damaged cellular membranes. Mice were sacrificed at designated time points and tissue was harvested for analysis. The uninjured quadriceps muscle on the contralateral leg was also harvested for use as controls.

Histology. Muscles were dissected from euthanized mice and fixed overnight at 4°C in 4% paraformaldehyde. The tissues were then washed in phosphate buffered saline (PBS), dehydrated in serial ethanol dilutions, and embedded in paraffin. 5μm sections were stained with hematoxylin and eosin or Masson's trichrome and imaged using CellSens (Olympus) software and an Olympus BX50 microscope.

The minimal Feret's diameter of muscle fibers containing centralized nuclei was manually measured on transverse hematoxylin and eosin stained sections using ImageJ software.

Bone marrow-derived macrophage culture, proliferation assay, and polarization. Bone marrow was harvested from femurs as described previously (Goncalves & Mosser, 2015). Bone marrow derived cells (BDMC) were plated at $4x10^5$ and cultured in M-CSF from L929-conditioned media, heat-inactivated FBS, and RPMI 1640 (Manzanero, 2012). Macrophage differentiation was verified by flow cytometric analysis of $F4/80^+$ stained cells. For the proliferation assay, $1x10^5$ cells were plated per well, 3 wells per experimental time point, in a 6-well non-treated plate. A partial media change was performed every 2 days to maintain concentration of M-CSF. Adherent cells were collected in PBS, stained with Trypan blue (Sigma-Aldrich), and counted using a hemocytometer and an Olympus CK40 microscope. To induce polarization, BMDM were plated at 36,000 cells/cm^2, 3 wells per condition, in a non-treated 24-well plate and

stimulated with 10ng/mL IFNγ 6 hours after plating (Saclier et al., 2017). 3 days later, cells were harvested, washed, and stained with F4/80 and Gr1. Polarization efficacy was assessed via flow cytometry.

mRNA isolation and quantitative real-time PCR. RNA was extracted from isolated *Mkx*$^{-/-}$ and *Mkx*$^{+/+}$ cells or whole quadriceps tissue with TRIzol (Life Technologies) according to manufacturer's protocol. Reverse transcription of 1μg of total RNA was performed using Superscript III (Invitrogen). Real-time quantitative PCR analysis of the cDNA was performed using qPCR MasterMix Plus w/o UNG (Eurogentec) on an ABI 7900HT quantitative Real-Time PCR machine. Results were analyzed with the standard delta cycle threshold method and were normalized to the transcription of *Gapdh* (Livak & Schmittgen, 2001).

Flow cytometry. Single-cell suspensions from muscle were prepared by collagenase II (Worthington Biochemical Corp.) digestion followed by staining with fluorochrome-conjugated antibodies (Gr1 Clone RB6-8C5: eBioscience 48-5931-82; F4/80 Clone BM8: Invitrogen 11-4801-82; CD11b Clone M1/70: Invitrogen 17-0112-81; Siglec-F Clone E50-2440 (RUO): BD BioSciences 552126; CD206 Clone C068C2: BioLegend 141701) and live dead stain (eBiosciences). Data acquisition was performed on a FACS Aria or Fortessa (BD Biosciences). UltraComp eBeads (Invitrogen) were used to generate single-stain controls. Data was analyzed using FlowJo software and gating strategies to discriminate against dead cells, debris, and doublets were utilized. M1 macrophages were defined as Gr1$^{low\text{-}med}$, F4/80^{+}, and CD11b^{+}.

Immune response challenge. *Salmonella enterica* serovar Typhimurium strain (χ3761) was inoculated from a freezer stock into a 5 ml culture of Luria-Bertani (LB) broth and grown statically at 37°C for 18 hours. The overnight statically grown cultures were inoculated at a 1:50 dilution into fresh pre-warmed LB broth and grown with gentle aeration at 37 °C until OD_{600}= 0.85-0.9. Cultures were centrifuged at 6000 x g for 15 minutes at room temperature and the pellet gently resuspended in 250µl of sterile PBS. The culture was brought up to 500µl with sterile PBS, serially diluted, and plated on LB agar to determine CFU per ml as the basis for determining the actual dose of the challenge strain. Mice were deprived of food and water for 6 hours immediately prior to oral inoculation behind the incisors with 20µl of the bacterial strain. Food and water were returned 30 minutes after oral inoculation. 5 mice each were inoculated with 10^2, 10^3, 10^5 and 10^7 CFU *S. enterica* to determine the degree of virulence. LD_{50} values were calculated by the method of Reed and Meunch.

Statistics. All experiments were performed using at least three biological replicates. Results were expressed as means ± SD. Student's t test and two-way ANOVA were performed in Microsoft Excel and $p \leq 0.05$ was considered significant ($*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$).

Results

Impaired muscle regeneration in the absence of MKX. Based on the demonstrated role of *Mkx* in muscle differentiation, skeletal muscle development was examined in mice homozygous for a null mutation in the *Mkx* loci ($Mkx^{-/-}$) (Anderson et al., 2012). Offspring from a heterozygous ($Mkx^{+/-}$) cross resulted in live births of $Mkx^{-/-}$

and *Mkx*$^{+/-}$ at normal Mendelian ratios. No differences were observed in the size of body wall and appendicular muscles by histological analysis (data not shown), indicating that *Mkx* is not required for the normal development of skeletal muscle.

A role for *Mkx* in adult mammalian myogenic processes was examined by inducing muscle repair in response to acute injury. Using the well-characterized cardiotoxin (CTX)-induced injury model, an acute injury induces an immediate inflammatory response followed by activation of satellite cells, differentiation of myotubes and fiber maturation over a 21 day period (Arnold et al., 2007). Quadriceps muscle was injected with CTX and tissue was harvested at 10 and 21 days post-injury (DPI) to morphologically assess muscle repair. At both timepoints, histological cross-sections of *Mkx*$^{-/-}$ and *Mkx*$^{+/+}$ quadriceps revealed muscle fibers with centralized nuclei indicating active repair (Figure 3). However, at 10 DPI the *Mkx*$^{-/-}$ muscle contained clusters of necrotic muscle fibers that are normally cleared by M1 macrophages in the first 3 days post-injury (Figure 3B, F). Qualitatively, there appeared to be a reduction in the diameter of the repaired fibers from the *Mkx*$^{-/-}$ muscle. Based on Masson's trichrome staining, the region of repair in the mutant muscle had a greater concentration of collagen (Figure 3F). To quantify the robustness of muscle repair, the minimal Feret's diameter of fibers with centralized nuclei was measured. At 10 DPI, the diameter of repairing fibers in the *Mkx*$^{-/-}$ muscle were approximately half the size of the repairing fibers in WT muscle (Figure 3G). By 21 DPI, the necrotic fibers were no longer present (Figure 3D) and the diameter of the fibers were more comparable to the wild type muscle (Figure 3H).

Collectively, this demonstrates that *Mkx* is required for efficient clearance of injured fibers and promotion of new fibers early in the repair process.

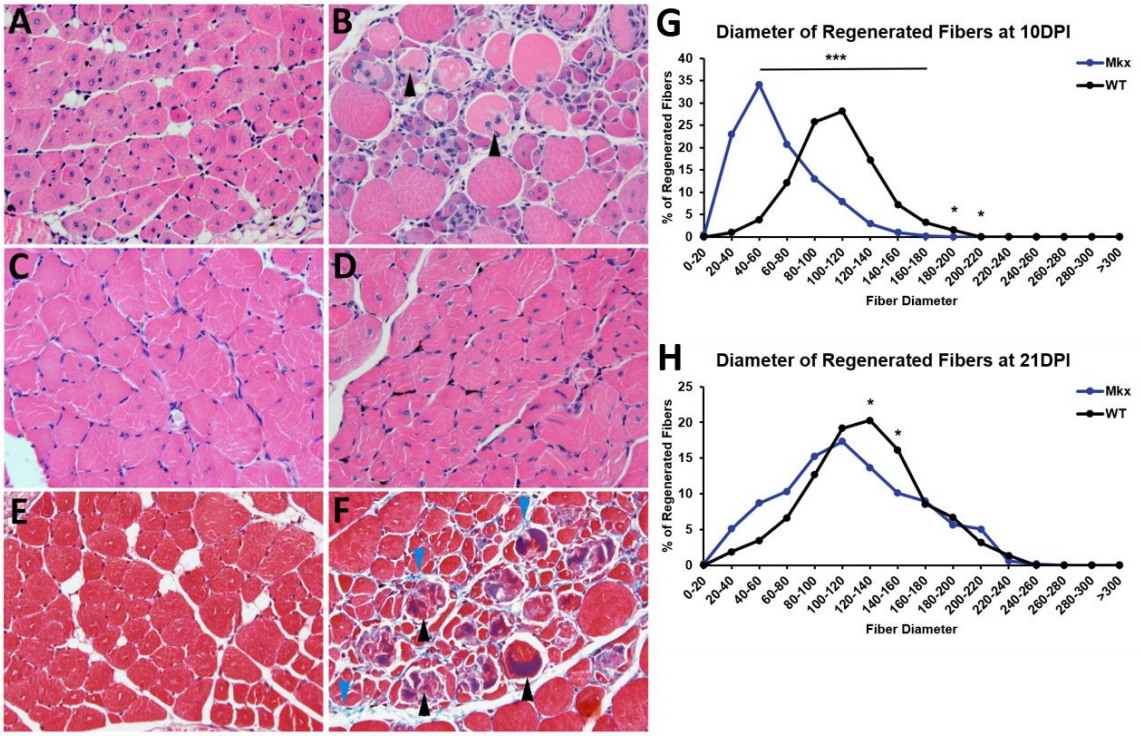

Figure 3. Delayed muscle regeneration in *Mkx*$^{-/-}$ following cardiotoxin (CTX) induced damage. (A) H&E stained transverse sections at 10 days post-injury (DPI) in WT and (B) *Mkx*$^{-/-}$ quadriceps. (C) H&E stained transverse sections at 21 days post-injury in WT and (D) *Mkx*$^{-/-}$ quadriceps. (E) Masson's trichrome stained transverse sections at 10 DPI in WT and (F) *Mkx*$^{-/-}$ quadriceps. (G) Distribution of regenerating fiber diameter in WT and *Mkx*$^{-/-}$ mice at 10 and (H) 21 DPI. Fiber measurements are displayed in pixel units. Black arrowheads denote necrotic fibers and blue arrowheads denote collagen deposition. Data are mean ± SD, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

Inflammatory cell recruitment to the site of muscle injury. The persistence of necrotic fibers at 10 DPI that were observed in the *Mkx*$^{-/-}$ muscle is phenotypically similar to what was previously described for a mutation in the macrophage chemoattractant CCL2 (Lu et al., 2011). This suggests that a failure of M1 macrophage infiltration at the site of damage could be responsible for the failure to clear necrotic fibers. To examine this, M1 macrophages were quantified via flow cytometric analysis in CTX-injured

muscle and contralateral control muscle at 2 DPI. This is a time when the pro-inflammatory response is expected to be at its peak. Cells were stained with fluorescently conjugated F4/80, CD11b and Gr1 to identify M1 macrophages. In CTX-injured *Mkx*$^{-/-}$ muscle, there were significantly fewer cells M1 macrophages (Gr1$^{low-med}$, F4/80^{+}, CD11b^{+}) in comparison with WT injured muscle (Figure 4B). The nearly absent population of Gr1$^{low-med}$, F4/80^{+}, and CD11b^{+} cells at 2 DPI supports the hypothesis that M1 macrophages are not substantially present in *Mkx*$^{-/-}$ muscle during the pro-inflammatory response. No significant differences in the amount of M2 macrophages or eosinophils were identified (Figure 4C, D). Immunohistochemical staining for the pan-macrophage marker F4/80 in injured muscle at 2 DPI confirmed a decrease in inflammatory cell recruitment (Figure 4E).

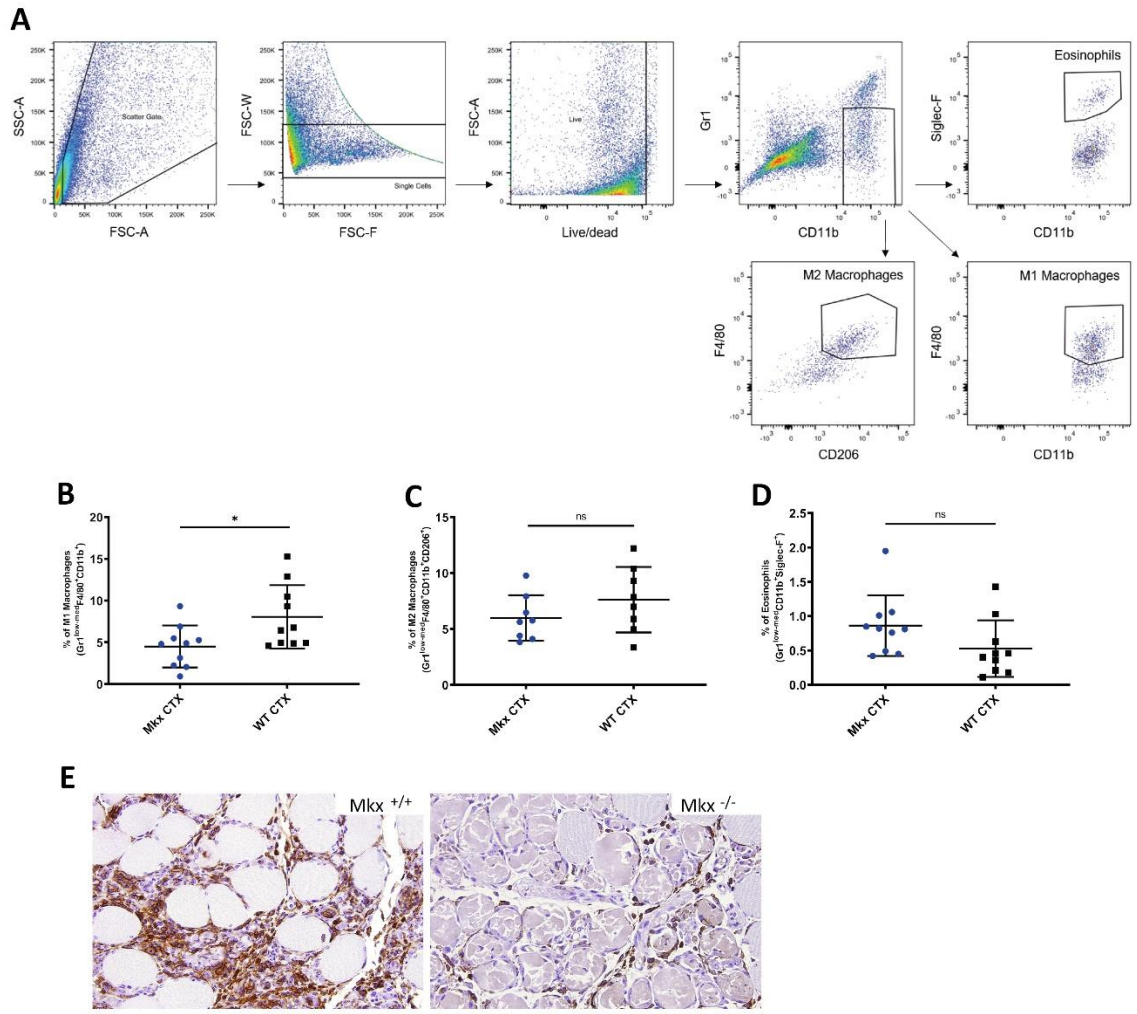

Figure 4. Deficit of M1 macrophages in *Mkx*$^{-/-}$ muscle after injury. Gating strategy for flow cytometry analysis of macrophage and eosinophil populations in muscle (A). Quantification of M1 macrophages (B), M2 macrophages (C) and eosinophils (D) at 2 DPI in *Mkx*$^{-/-}$ and WT muscle. Representative images of F4/80 immunohistochemical staining of injured muscle at 3 DPI (E). Data are mean ± SD, *$p \leq 0.05$.

Compromised innate immune response in *Mkx*$^{-/-}$ mice. M1 macrophages are a central component of the innate immune response for both tissue repair and infection (Benoit et al., 2008). To assess whether *Mkx* regulation of M1 macrophages is specific to muscle repair or part of a systemic recruitment, *Mkx*$^{-/-}$ mice were challenged with the bacteria, *Salmonella enterica* serovar Typhimurium. *Mkx*$^{-/-}$ and WT mice were inoculated

with four serial doses of *S. enterica* and virulence was determined by the LD_{50}, the bacterial dose causing lethality in 50% of the exposed population. It was observed that over a 17-day incubation period, *Mkx*$^{-/-}$ mice were more sensitive to lower doses of *S. enterica*, as compared to WT mice (Figure 5). At a dose of 10^2 colony forming units (CFU), WT mice were able to resolve the infection while 40% of the *Mkx*$^{-/-}$ mice succumbed to infection. Overall, the LD_{50} for *Mkx*$^{-/-}$ mice was 5.3 x 10^2 CFU, in comparison with an LD_{50} of 3.7 x 10^4 for WT mice. This indicates a 70-fold increase in susceptibility of *Mkx*$^{-/-}$ mice to *S. enterica* and suggests *Mkx* is necessary for the activation of the pro-inflammatory immune response.

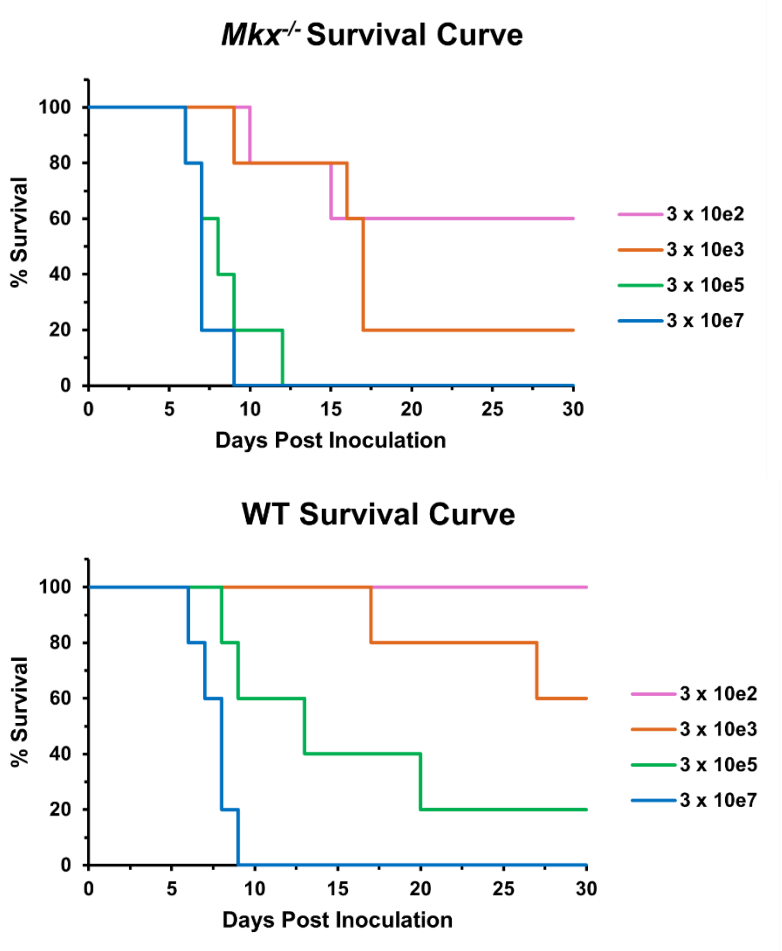

***Figure 5*.** Increased susceptibility to bacterial infection. Kaplan-Meier survival curves for wildtype and *Mkx*$^{-/-}$ mice following inoculation with 10^2, 10^3, 10^5 and 10^7 CFU *S. enterica*. The Reed and Meunch method was used to calculate LD_{50} values. (n=5 mice per dose for each wildtype and *Mkx*$^{-/-}$).

Proliferation and polarization of *Mkx*$^{-/-}$ bone marrow-derived macrophages (BMDMs) in vitro. To address whether the reduced population of M1 macrophages is intrinsic or based on environmental cues, BMDMs from WT and *Mkx*$^{-/-}$ mice were isolated from the femur and tested for their capacity to proliferate and polarize. Bone marrow derived cells were cultured in the presence of macrophage colony-stimulating factor (M-CSF), which induces the proliferation and maturation of macrophages. To confirm that the BMDCs had differentiated into macrophages, cells were first analyzed for F4/80 expression through flow cytometry. Starting at 4 days of culture and continuing every 2 days over a two-week period, independent dishes of cells were removed, stained with Trypan blue, and live cells were counted. *Mkx*$^{-/-}$ macrophages exhibited a significantly reduced rate of proliferation as compared to WT cells (Figure 6).

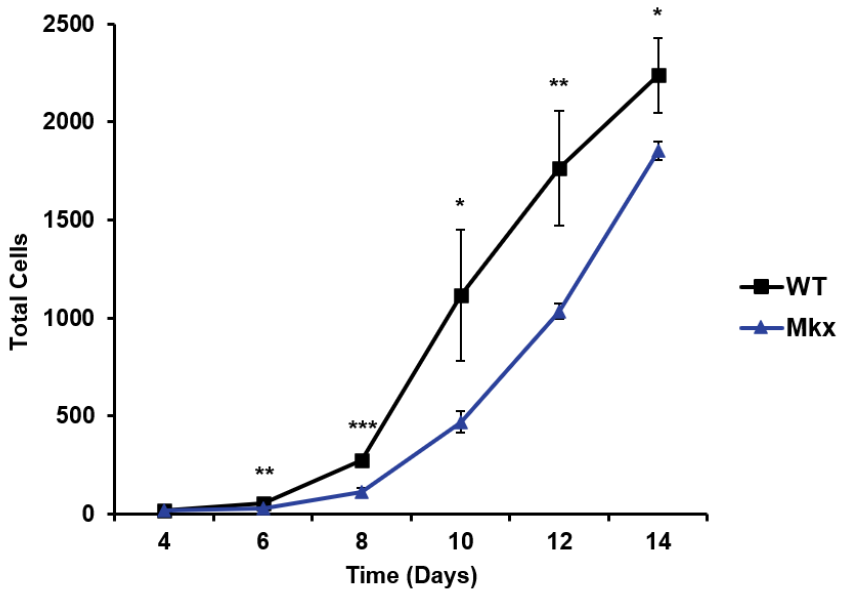

Figure 6. Delayed proliferation and proficient polarization capacity of *Mkx*$^{-/-}$ bone marrow-derived macrophages (BMDM). (*A*) Proliferation of wildtype and *Mkx*$^{-/-}$ BMDM in culture. The number of cells were determined by manual counting under a microscope. Data are mean ± SD, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

Polarization of BMDMs to the M1 pro-inflammatory subtype was examined in culture by a 3-day treatment with INFγ (10ng/mL) (Saclier et al., 2017). INFγ-treated and untreated cells were analyzed using flow cytometry for the presence of the M1 macrophage-specific surface marker, Gr1. Treatment of *Mkx*$^{-/-}$ BMDMs lead to a clear shift from Gr1^{-} to Gr1^{+} cells, indicating these cells have the capacity to polarize (Figure 7). This data suggests that *Mkx* does not play an intrinsic role in M1 macrophage polarization and points to a role to regulating pro-inflammatory signals directing macrophage infiltration.

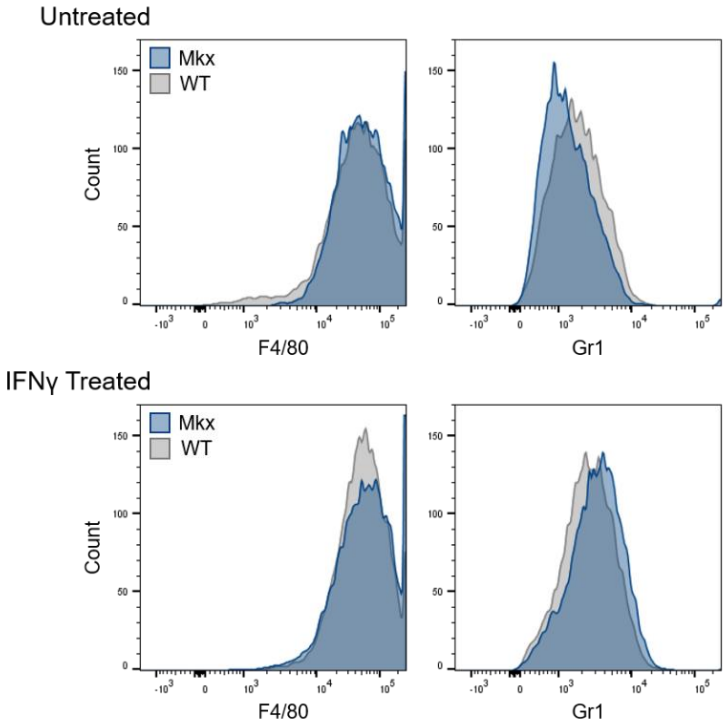

Figure 7. Proficient Polarization Capacity of *Mkx*$^{-/-}$ Bone Marrow-Derived Macrophages. Representative flow cytometry analysis of F4/80 and Gr1 surface expression from wildtype and *Mkx*$^{-/-}$ BMDM following treatment with 10ng/mL IFNγ. Grey histogram peaks denote wildtype cells and blue histogram peaks denote *Mkx*$^{-/-}$ cells.

Altered expression of inflammatory cytokine expression in *Mkx*$^{-/-}$ skeletal muscle following injury. The role of *Mkx* in regulating the expression of cytokines and chemokines associated with the innate immune response was examined 1, 2, 3, and 5 days post-injection of CTX in the quadriceps. As signaling factors are expressed by both resident macrophages and muscle tissue, total RNA was isolated from all cells at the site of injury and qRT-PCR was used to quantify transcription differences between *Mkx*$^{-/-}$ and WT mice. RNA from the quadriceps on the contralateral leg was used as an internal control for each genotype.

The pro-inflammatory chemokine *Ccl2*, which is exhibits chemotactic activity for monocytes, was downregulated at 1 and 3 DPI (Figure 8). Similarly, *Tnfα* and *Ifnγ,*

cytokines secreted by M1 macrophages, were also reduced at 1 and 3 DPI. The downregulation of these pro-inflammatory cytokines results in a dampened inflammatory response that is insufficient to clear necrotic fibers and allow for robust repair to occur. While increased expression of *Ccl2*, *Tnfα,* and *Ifnγ* was evident at 2 DPI, in most cases it was not significant and expression rapidly decreased by 3 DPI. The peak in expression at 2 DPI is likely due to a delayed, though still blunted, inflammatory response in *Mkx*$^{-/-}$ tissue. *Tgfβ* and *Il-10*, anti-inflammatory cytokines released by M2 macrophages, were both significantly downregulated at 1, 2, and 3 DPI. By 5 DPI, expression of all inflammatory markers was comparable between WT and *Mkx*$^{-/-}$ tissue, with no significant changes in expression. The downregulation of these inflammatory markers points to a possible mechanism in which *Mkx*$^{-/-}$ mice fail to mount an adequate pro-inflammatory immune response during the early stages of muscle repair.

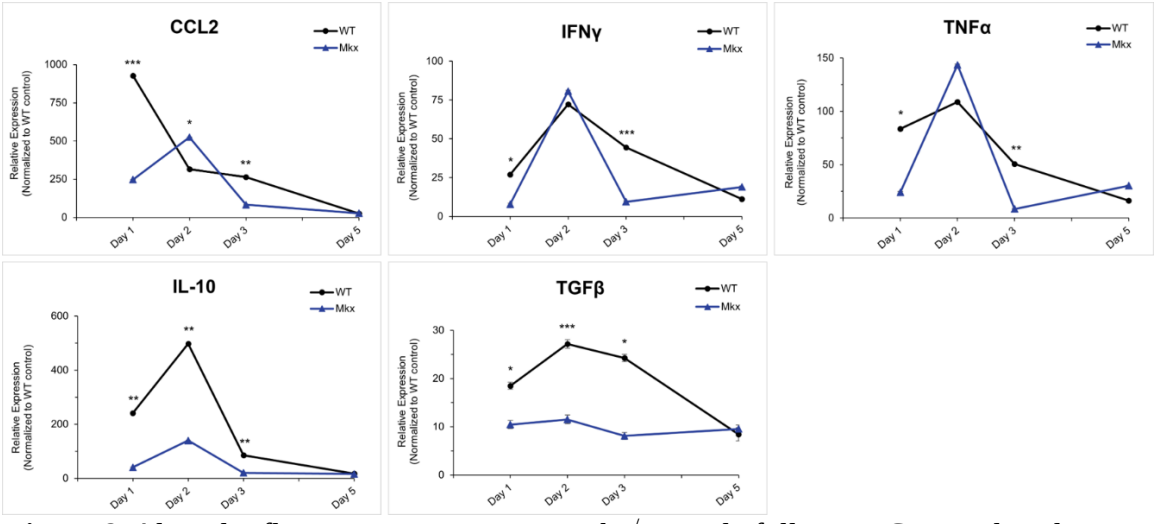

Figure 8. Altered inflammatory response in *Mkx*$^{-/-}$ muscle following CTX-induced injury. mRNA levels of pro-inflammatory genes *Ccl2*, *Tnfα*, *Ifnγ*, and anti-inflammatory genes *Tgfβ* and *Il-10* analyzed by quantitative real-time PCR. (n=5 at day 1, n=9 at day 2, n=4 at day 3, n=3 at day 5). Data are mean ± SD, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

Discussion

Mkx is a member of the TALE superclass of atypical homeobox genes that has been reported to play an important role in development of tendons and ligaments (Ito et al., 2010; W. Liu et al., 2010; Nakamichi et al., 2016). Studies in cell culture have demonstrated *Mkx* inhibits differentiation of myocytes, osteocytes, chondrocytes, and adipocytes. This study reports a novel role for the gene in efficient repair of skeletal muscle in response to acute injury. Despite the increasing scope of known contributors in muscle repair, the essential role of the macrophage is continually demonstrated (Baht et al., 2020; Chazaud, 2020; Stepien et al., 2020). The delay in removal of necrotic muscle fibers and reduced recruitment of M1 macrophages to the site of the injury immediately after injury predicts a non-myogenic role in initiation of the innate immune response. A delay in growth of repairing muscle fibers raises the possibility of a separate role for *Mkx* in regulating the myogenic pathway as well.

The deficiency in $Mkx^{-/-}$ muscle repair appears to be linked to the activation of the innate immune response. Following an acute injury in muscle, key cytokines (i.e. CXCL1, IL-1, IL-8, and CCL2) are immediately released by neutrophils and resident macrophages to recruit circulating monocytes to site of damage (Fujishima et al., 1993; Lu et al., 2011). M1 macrophages, which derive from their monocyte precursor, are essential for the clearance of necrotic fibers. Genetic ablation of *Ccl2*, or its receptor *Ccr2*, impairs macrophage infiltration and phagocytosis at the site of induced muscle injury (Lu et al., 2011). The histopathology is similar to that of $Mkx^{-/-}$ in that there is a delay in clearance of necrotic fibers and smaller regenerating myofibers. Evaluation of

the pattern of *Ccl2* expression in damaged *Mkx*$^{-/-}$ muscle after injury reveals a delay in expression, predicting a role for *Mkx* in regulating the cytokine's transcription. In addition to CCL2, TNFα and IFNγ produced by M1 macrophages are necessary to elicit a robust pro-inflammatory response. IFNγ expression increases simultaneously with the influx of neutrophils, macrophages, and *MyoD*$^{+}$ satellite cells in damaged muscle. Notably, *Ifnγ*$^{-/-}$ mice exhibit diminished muscle repair, impaired macrophage function, and increased development of fibrosis (Cheng et al., 2008). TNFα expression is also coupled with the early inflammatory response and its production is almost exclusively by neutrophils and macrophages (Collins & Grounds, 2001). Together, IFNγ, and TNFα are also able to synergize in the activation of M1 macrophages (Mills et al., 2000). It is noteworthy that both *Ifnγ,* and *Tnfα* were significantly reduced at 1 DPI and 3 DPI after muscle injury in *Mkx*$^{-/-}$ mice. This predicts that *Mkx* participates in regulating the timing of pro-inflammatory cytokine expression in response to injury.

The transition from an M1-biased to an M2-biased environment is accompanied by increased expression of anti-inflammatory cytokines *Il-10* and *Tgfβ*. While a difference in the number of M2 macrophages at 2 DPI was not found, *Il-10* and *Tgfβ* were significantly downregulated at 1, 2, and 3 DPI. This alteration in expression is likely due to the great plasticity and diversity of macrophage phenotypes, as individual macrophages have been found to express both pro- and anti-inflammatory cytokines concurrently (Lemos et al., 2015). It is also possible that the reduction in signaling critical for establishing a pro-inflammatory environment consequently impacted signaling associated with the anti-inflammatory response. The expression of *Tgfβ* has been shown

to be regulated by *Mkx* in tenocytes (Kayama et al., 2016) and other research has demonstrated that MKX is capable of binding to the *Tgfb2* promoter (Huanhuan Liu, Zhang, et al., 2014). These results indicate that MKX and TGFβ may function in a positive feedback loop to increase ECM strength during tendon remodeling (Subramanian & Schilling, 2015). Recently, interstitial tenocytes residing within skeletal muscle have been identified, though their function in muscle repair is not yet understood (De Micheli et al., 2020; Dell'Orso et al., 2019; Giordani et al., 2019), these studies provide evidence of another possible mechanism in which *Mkx* might regulate muscle repair.

The innate immune response associated with skeletal muscle repair is shared with sterile tissue repair and response to infection. To determine whether the reduced pro-inflammatory response is specific to muscle, $Mkx^{-/-}$ mice were infected with *S. enterica*, a gram-negative bacterium that invades the mucosa of the gastrointestinal (GI) tract and causes an acute inflammatory reaction. A 70-fold increase in susceptibility to *S. enterica* is consistent with a broader role of *Mkx*, though the cell lineages involved are not clear. *S. enterica* infection can occur through multiple pathways, including via M cell mediated transcytosis at the Peyer's patches (Broz et al., 2012). While it is hypothesized that the increase in susceptibility is due to *Mkx* involvement in the induction of pro-inflammatory M1 macrophages, it is also possible that it has additional roles in other cell types (e.g. M cells) that were not investigated.

The cells types regulated by *Mkx* during the pro-inflammatory response have yet to be determined. Among cells associated with the innate immune response *Mkx* has been found to be expressed at the highest levels in eosinophils, a specialized type of white

blood cell, with an expression profile highly similar to that of the eosinophil receptor *Ccr3* (de Graaf et al., 2016). While eosinophils are largely implicated in allergic response and parasitic infection, their role in skeletal muscle is only beginning to unfold. Heredia et al. (2013), demonstrated that eosinophils are rapidly recruited to the site of muscle damage where they activate the pro-regenerative function of FAPs via secretion of IL-4. Mice that are deficient in eosinophils fail to regenerate skeletal muscle post injury, likely due to the loss of eosinophil-secreted factors such as IL-4 (Heredia et al., 2013). In the DMD mouse model (*mdx*), eosinophils are present in the muscle at high concentrations (Cai et al., 2000) and have been shown to promote lysis of muscle cells through the release of major basic protein-1 (MBP-1) (Wehling-henricks et al., 2008). When eosinophils are depleted using anti-CCR3 in *mdx* mice, muscle cell lysis is reduced. Further, when the expression of MBP-1 is ablated, the accumulation of fibrosis in muscle and heart is greatly decreased (Wehling-henricks et al., 2008). The essential role of eosinophils in mediating a healthy inflammatory response during muscle repair along with the high expression of *Mkx* in eosinophils suggests a possible mechanism in which *Mkx* expression in eosinophils modulates the local inflammatory response following muscle damage. Further research into the precise function of *Mkx* in eosinophils could provide valuable insight into potential therapeutic approaches aimed at dampening or modulating inflammation, such as those critical for the treatment of muscular dystrophies.

CHAPTER 4

PROMOTING EFFICIENT MUSCLE REPAIR IN DUCHENNE MUSCULAR DYSTROPHY PATIENTS THROUGH EOSINOPHIL REGULATION

Abstract

Duchenne Muscular Dystrophy (DMD) is characterized by muscle weakness and wasting during early childhood and a loss of ambulation and death by early adulthood. Chronic inflammation associated with repeated cycles of muscle fiber instability leads to fibrosis, which is a primary cause for loss of muscle function. Despite identification of the genetic basis for DMD over three decades ago, there is still no known cure or efficacious treatment for DMD. With this, parsing the immune cells types and signaling pathways that facilitate efficient muscle repair has been a priority, though the majority of research has focused on the role of macrophages. Eosinophils have been identified as critical mediators in the signaling required for healthy muscle repair and further regulate inflammation in mice lacking dystrophin, though the mechanism remains poorly understood. Using an inducible eosinophil-deficient mouse model, iPHIL, depletion of eosinophils prior to muscle injury and during the first 7 days of repair significantly increased the diameter of regenerated muscle fibers, indicating enhanced robustness of repair in the absence of eosinophils. In the DMD mouse model that closely mimics the human disease pathology, *mdx*$^{-/-}$*utrn*$^{-/-}$, eosinophil depletion from 3 to 12 weeks of age statistically increased the survival rate as well as the fiber diameter in the diaphragm muscle. These studies provide the foundation for important research in the modulation of

eosinophils in response to acute and chronic muscle injuries to promote healthy muscle regeneration.

Introduction

Duchenne Muscular Dystrophy (DMD), an X-linked disease affecting approximately 1 in 3,500 males, is characterized by severe and progressive muscle atrophy that ultimately leads to death by early adulthood (Emery, 1991). The absence of a functional Dystrophin protein, which links the cytoskeleton to the extracellular matrix (ECM), disrupts muscle cell membrane integrity causing patients to suffer from chronic inflammation and repeated cycles of muscle repair due to an increased susceptibility to muscle fiber damage. The continuous rounds of inflammation and muscle repair eventually lead to accumulation of fibrosis and loss of muscle function. Despite identification of the genetic basis for DMD over three decades ago (Hoffman et al., 1987), there is still no known curative treatment for DMD and the standard care treatment consists of broad-spectrum immunosuppressants.

Dystrophic muscle is marked by acute lesions that are infiltrated by a host of inflammatory cells, including neutrophils, macrophages, mast cells, eosinophils, and $CD8^+$ cytotoxic T-lymphocytes (Tidball et al., 2018). Of these, macrophages have been identified as the primary effectors of muscle membrane damage and are well-researched in the context of muscle repair and muscular dystrophy (Villalta et al., 2008). While eosinophils are recognized as significant contributors to repair in other tissues (Gieseck et al., 2017) and are present in elevated numbers in dystrophic muscle lesions (Cai et al., 2000), their role in muscle repair is less well-studied. Previous research has shown that

eosinophil deficiency leads to a loss of IL-4 signaling and subsequent failed muscle regeneration, demonstrating a necessity for eosinophil mediated signaling during healthy repair (Heredia et al., 2013). In the context of muscular dystrophy, research using the mildly symptomatic dystrophin-deficient mouse (*mdx*), has identified a role for eosinophils in contributing to muscle cell lysis through the release of major basic protein (MBP-1). When eosinophils are depleted using anti-CCR3 in *mdx* mice, muscle lysis is greatly reduced. Additionally, genetic ablation of MBP-1 leads to a reduction in the level of fibrosis present in dystrophic skeletal muscle and heart (Wehling-henricks et al., 2008). These studies point towards an under-appreciated contribution of eosinophils in muscle inflammation and provide an avenue for the development of potential therapeutics to treat muscular dystrophy.

In this research, the contribution of eosinophils in healthy muscle repair was investigated through utilization of the inducible eosinophil-deficient mouse model, iPHIL. In this model, the complete open reading frame of the human diphtheria toxin receptor (DTR) has been inserted in the eosinophil peroxidase (EPX) gene locus, which renders naturally DT-resistant mouse eosinophils sensitive to DT administration (Jacobsen et al., 2014). Ablation of eosinophils prior to acute muscle injury and during the first 7 days of repair significantly increased the diameter of regenerated muscle fibers, suggesting that eosinophils negatively impact the growth and maturation stages of muscle regeneration.

To further characterize the role of eosinophils in muscle repair and test the possible therapeutic effect of eosinophil depletion on dystrophic muscle, *mdx*/*utrn*

double-knockout (dKO) mice were utilized. *mdx*$^{-/-}$*utrn*$^{-/-}$ mice, lacking both Dystrophin and its homolog Utrophin, exhibit a profound dystrophic phenotype early in life that is characterized by repeated muscle fiber degeneration, accumulation of fibrosis, diminished capacity of muscle regeneration, severe inflammation, and death by 20 weeks of age (Deconinck et al., 1997; Isaac et al., 2013). TRFK-5, an IL-5 neutralizing antibody, is capable of depleting nearly 98% of blood eosinophils for a half-life of ~2 weeks and has demonstrated therapeutic effects in the treatment of eosinophilic asthma (Garlisi et al., 1999; Masterson et al., 2015). In this research, the therapeutic effect of TRFK-5 was investigated in *mdx*/*utrn* dKO mice. IL-5 neutralization over the course of the first 9 weeks following weaning significantly increased the lifespan of *mdx*/*utrn* dKO mice, accompanied by an increase of fiber diameters in the diaphragm.

Materials and Methods

Mice and genotyping. iPHIL mice on a C57BL/6 mixed background have been previously reported (Jacobsen et al., 2014). Mice were maintained in ventilated micro-isolator cages housed in a pathogen-free animal facility at the Mayo Clinic in Arizona under low barrier conditions, prior to being transported to Arizona State University (ASU) for subsequent experiments. All protocols and studies involving animals were performed in accordance with National Institutes of Health and Mayo Foundation institutional guidelines.

Mdx/utrn (stock *Utrn*^{tm1Ked} *Dmd*^{mdx}/J) mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and were bred and used in accordance with the Institutional Animal Care and Use Committee (IACUC) at ASU. Mdx/utrn mice were

genotyped as previously described by The Jackson Laboratory (protocol 21940). During the course of treatment, mice were given softened food daily to aid in weight maintenance.

All mice were housed in sterile, pathogen-free isolation cages and monitored by the Department of Animal Care and Technologies (DACT) at ASU.

Diphtheria toxin administration. Diphtheria toxin (DT, (Sigma)) was administered to mice (control animals received saline vehicle alone) by intra-peritoneal (i.p.) injection (1.5ng/µl) at a final dosage of 15ng/gram body weight, as described previously (Jacobsen et al., 2014). DT was administered on days -1, 0, and every 2 days following. Eosinophil depletion was confirmed by blood smears, immunohistochemistry (ICH) for major basic protein (MBP-1), and flow cytometry using the Siglec-F marker.

Anti-TRFK-5 administration. Anti-TRFK-5 (IgGk1 clone) antibody (BioXCell) was administered via an i.p. injection, at a concentration of 7ug/gm of body weight every 5 days for the duration of the treatment (a total of 9 weeks). In control mice, IgGk1 control antibodies (BioXCell) were i.p. injected.

Muscle injury. Controlled injury of the right quadriceps of iPHIL mice at 3 months of age was induced by injection of 50µl of cardiotoxin (CTX, 10µM) (Latoxan). Mice were sacrificed at designated time points and tissue was harvested for analysis. The uninjured quadriceps muscle on the contralateral leg was also harvested for use as controls.

Histology. Muscles were dissected from euthanized mice and fixed overnight at 4°C in 4% paraformaldehyde. The tissues were then washed in phosphate buffered saline

(PBS), dehydrated in serial ethanol dilutions, and embedded in paraffin. 5μm sections were either stained with eosin and hematoxylin or underwent immunohistochemistry with an antibody to major basic protein (MBP-1).

The minimal Feret's diameter of muscle fibers was manually measured on transverse hematoxylin and eosin stained sections using ImageJ software.

Flow cytometry. Single-cell suspensions from muscle were prepared by collagenase II (Worthington Biochemical Corp.) digestion followed by staining with fluorochrome-conjugated antibodies (Gr1 Clone RB6-8C5; F4/80 Clone BM8; CD11b Clone M1/70; Siglec-F Clone E50-2440 (RUO); CD45.2) and live dead stain. Data acquisition was performed on a Fortessa (BD Biosciences). UltraComp eBeads (Invitrogen) were used to generate single-stain controls. Data was analyzed using FlowJo software and gating strategies to discriminate against dead cells, debris, and doublets were utilized.

Statistics. All experiments were performed using at least three biological replicates. Results were expressed as means ± SD. Student's t test and two-way ANOVA were performed in Microsoft Excel and $p \leq 0.05$ was considered significant ($*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$).

Results

Eosinophil depletion in injured muscle. To define the contribution of eosinophils following acute muscle damage, eosinophils were depleted via administration of DT in the iPHIL mouse model at days -1, 0, and every 2 days following. An acute muscle injury was induced on day 0 using the well-defined cardiotoxin (CTX)-induced

injury model, in which a local inflammatory response is immediately initiated and muscle regeneration is completed by 21 days (Arnold et al., 2007). Muscle was harvested and evaluated during the peak of the pro-inflammatory phase of muscle repair (3 days post-injury) and near the end of the anti-inflammatory phase (7 days post-injury). Eosinophil depletion at 3 days post-injury was confirmed by IHC using the eosinophil-specific antibody major basic protein (MBP-1) (Figure 9). In control iPHIL mice, receiving the saline vehicle alone, eosinophils are seen infiltrating into the site of muscle damage, as shown by the presence of MBP-1 staining. Conversely, in mice receiving DT, eosinophil infiltration is nearly nonexistent.

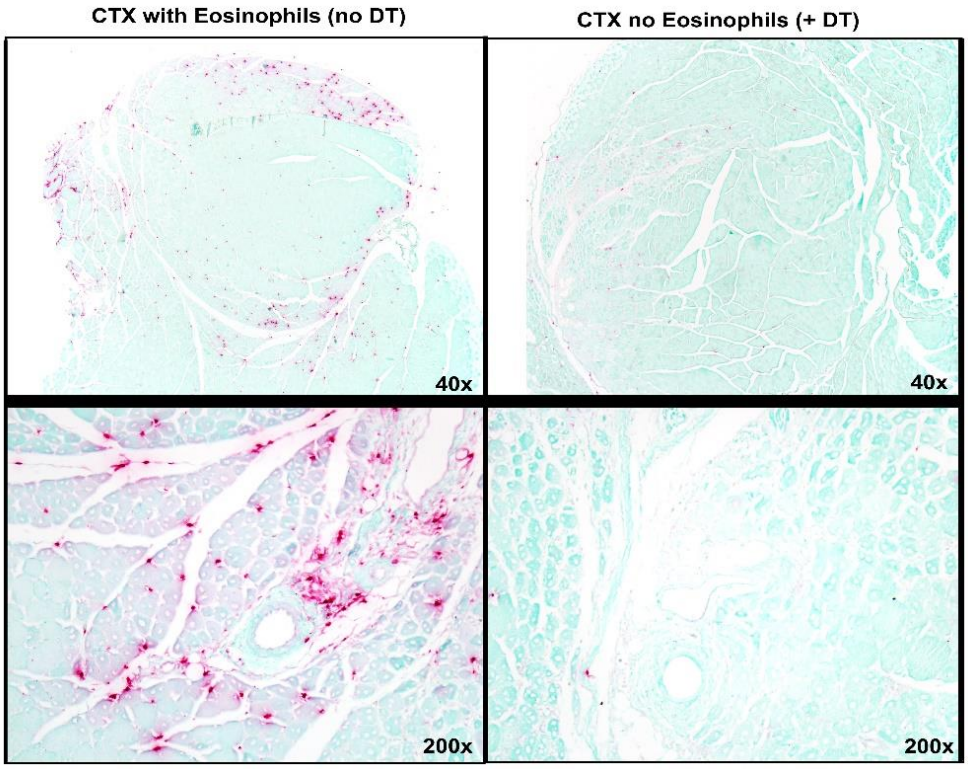

Figure 9. Eosinophils are significantly depleted by DT injection at day 3 post-CTX injury. Quadriceps muscle from mice treated with CTX were fixed, embedded, then underwent IHC with an antibody to major basic protein (MBP-1) (red) to identify eosinophils. Representative images at 40X and 200X magnification show eosinophils

infiltrating the muscle in mice without DT and near complete absence of eosinophils in mice with DT.

Further validating and quantifying the efficacy of eosinophil depletion, flow cytometric analysis of eosinophil infiltration at 2-3 and 7 days post-injury revealed that eosinophils ($CD11b^{+}F4/^{80med}Gr1^{low\text{-}med}Siglec\text{-}F^{+}$) comprised a significantly lower population of $CD45.2^{+}$ cells per quadricep in DT-treated mice, as compared with control mice (<1% and ~4%, respectively) (Figure 10B). The total number of eosinophils per quadricep at days 2-3 and 7 post-injury were also significantly lower in mice receiving DT. Interestingly, the total number of eosinophils in control mice decreased between days 2-3 and day 7, indicating an influx of eosinophils during the pro-inflammatory phase and subsequent decline during the anti-inflammatory phase (Figure 10C).

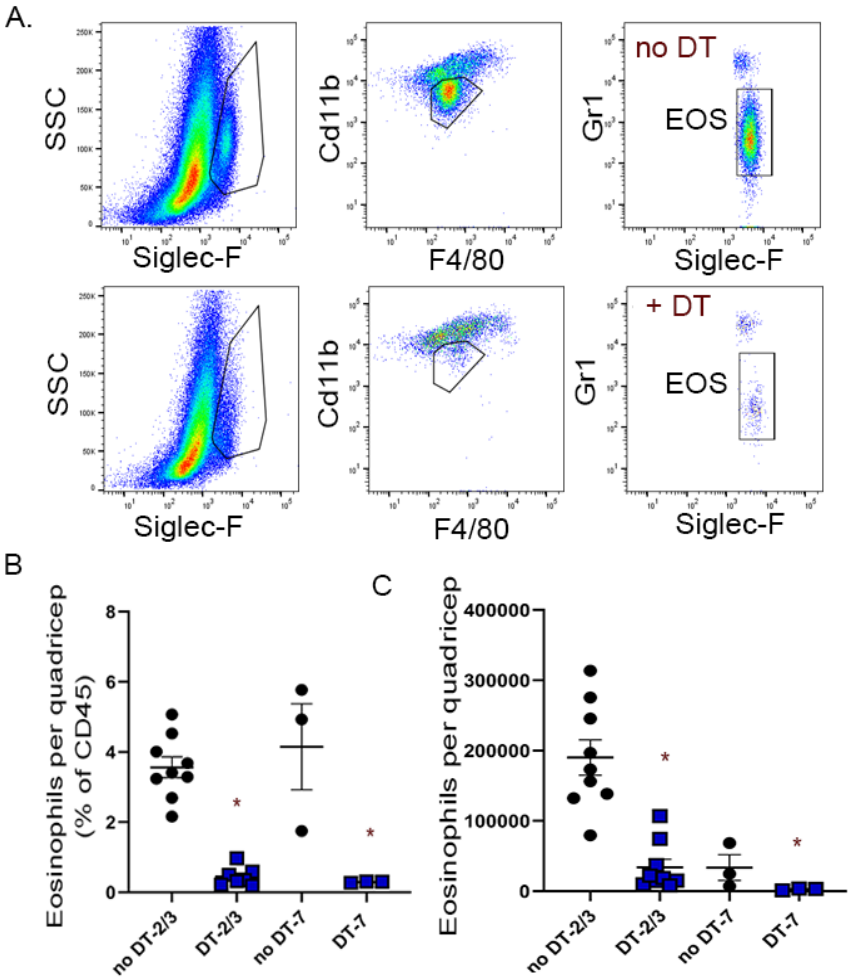

Figure 10. Eosinophils are significantly depleted by DT injection at days 2 and 3 post-CTX injury. (A) Flow cytometry gating after live/dead and CD45.2+. Top panel shows mice without DT treatment and bottom with DT treatment from 2 days post-CTX injury. (B) Percent eosinophils in quadriceps muscle out of CD45.2+ cells for days 2-3 and day 7 experiments. (C) Total eosinophils per individual quadriceps for same experimental groups. (n=3-8 mice). *=$p<0.05$.

Eosinophil depletion increases fiber diameter following injury. The efficiency of muscle repair in eosinophil-depleted iPHIL mice was evaluated at 7 days post-injury, after the influx of pro-inflammatory cells has subsided and during which time myotubes are undergoing terminal differentiation and growth. Histological assessment revealed an increase in the diameter of newly regenerated muscle fibers, as indicated by the presence

of centralized nuclei, in eosinophil-depleted mice (Figure 11). The increase in regenerated fiber diameters suggests that hypertrophic growth associated with this timepoint in muscle repair is at least in part regulated by eosinophils. Further, eosinophil depletion throughout the peak of both the pro- and anti-inflammatory phases appears to be beneficial to fiber regeneration.

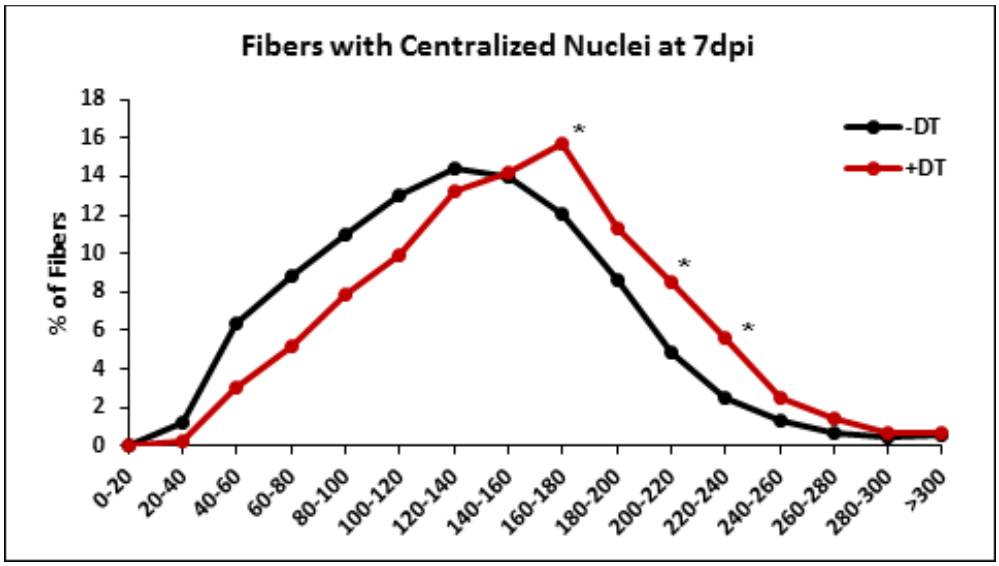

Figure 11. Administration of DT to selectively deplete eosinophils increased the diameter of newly regenerated muscle fibers at 7dpi. Distribution of fiber diameter of newly regenerated fibers 7-days-post CTX-induced injury in quadriceps muscle with and without administration of DT to deplete eosinophils. Statistical analysis performed using student's t-test. (-DT n=4; +DT n=5). *$p<0.05$.

Increased lifespan of mdx/utrn dKO mice following eosinophil depletion. The ability of eosinophil depletion to slow or suppress disease progression in a severely affected mouse model of muscular dystrophy was investigated through weekly administration of anti-IL-5 (TRFK-5) antibody in *mdx*/*utrn* dKO mice. Eosinophils were depleted for a total of 9 weeks, starting immediately after weaning at 3 weeks of age. Mice receiving anti-IL-5 exhibited a significantly increased lifespan, as compared to IgG control treated mice (Figure 12). After 5 weeks of treatment, 100% of anti-IL-5 treated

mice had survived, whereas only approximately 50% of IgG-treated mice had survived. By the 9th week of treatment, approximately 75% of anti-IL-5 treated mice had survived, as compared with 30% of IgG-treated mice. These results implicate eosinophils in contributing towards the dystrophic pathology that ultimately leads to premature death and confirm the therapeutic potential of anti-IL-5 in slowing disease progression.

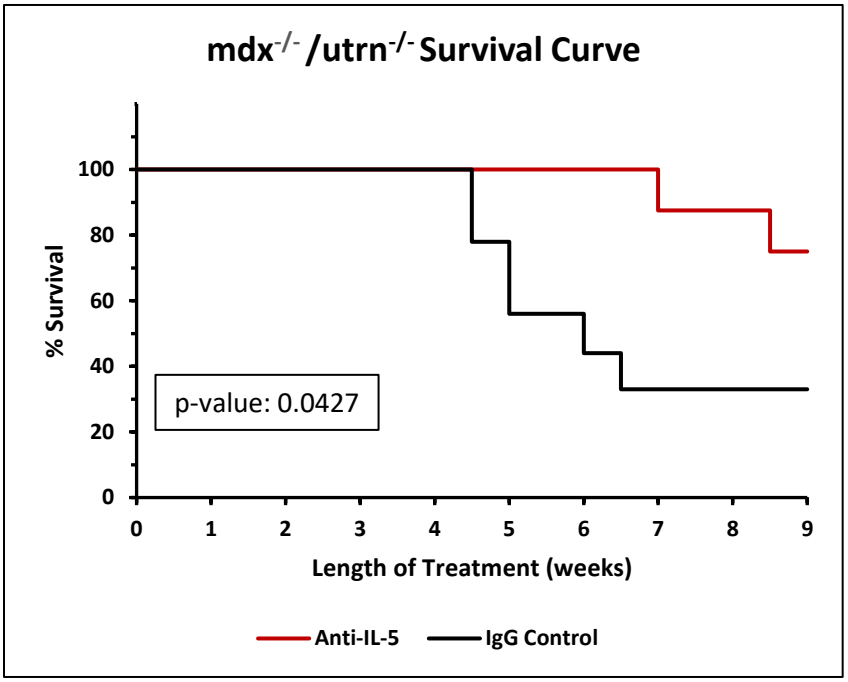

Figure 12. Treatment with anti-IL-5 statistically increased the survival rate of *mdx*$^{-/-}$*utrn*$^{-/-}$ mice. Kaplan-Meier survival curve and log-rank test of anti-IL-5 and IgG control treatment groups combined from two experimental treatment rounds. (Anti-IL-5 n=8; IgG control n=9).

Increased fiber diameters in diaphragm of *mdx*/*utrn* dKO by anti-IL-5. As respiratory failure is the leading cause of death in muscular dystrophy patients, alongside heart dysfunction (Lo Mauro & Aliverti, 2016), the morphology and distribution of fiber diameters in the diaphragm were evaluated. Histological assessment revealed no significant difference in fibrotic tissue accumulation between anti-IL-5 and IgG-treated *mdx*/*utrn* dKO mice (data not shown). The distribution of fiber diameters in the

diaphragm of anti-IL-5 treated mice was shifted towards larger fibers, in comparison to IgG-treated mice (Figure 13). Anti-IL-5 treatment improved the fiber diameter of dKO mice as the number of smaller fibers were significantly decreased and larger fibers were significantly increased. This data shows that eosinophil depletion improves the growth and maturation of dystrophic muscle fibers in the diaphragm.

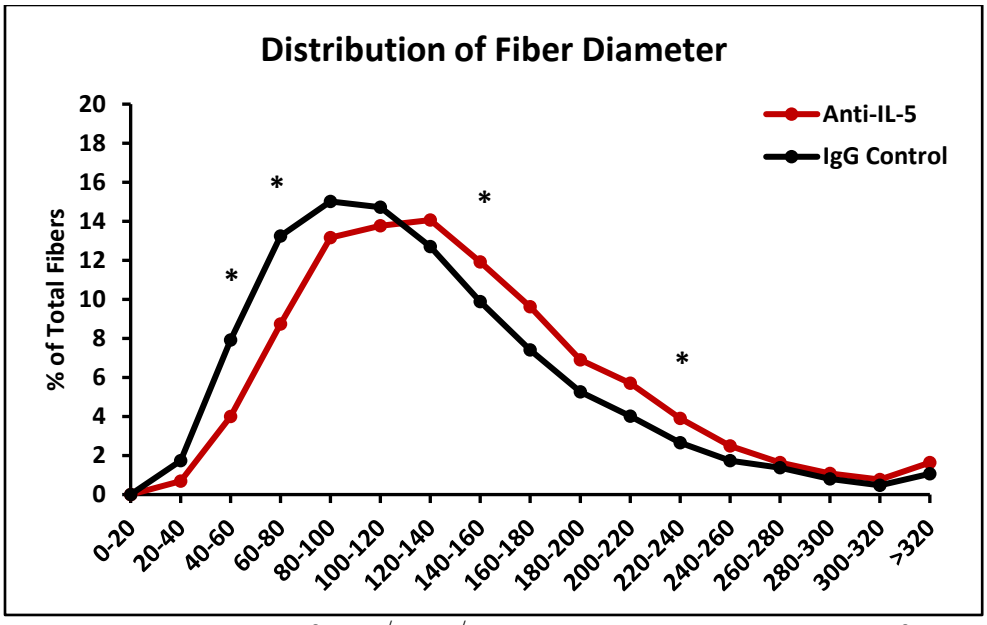

Figure 13. Treatment of *mdx*$^{-/-}$*utrn*$^{-/-}$ mice with anti-IL-5 antibody shifted the distribution of fiber diameter towards larger fibers**.** Diaphragm fiber measurements were performed by 3 individuals and averaged. Statistical analysis performed using student's t-test. (Anti-IL-5 n=4; IgG control n=5).

Discussion

This study aimed to characterize the role of eosinophils in muscle repair following acute and chronic muscle damage. To this end, two different mouse models were used to address each type of damage: (1) inducible eosinophil-deficient iPHIL and, (2) dystrophin and utrophin double-knockout (*mdx*$^{-/-}$*utrn*$^{-/-}$). Eosinophils were selectively depleted through the administration of DT in iPHIL mice and anti-IL-5 in *mdx*$^{-/-}$*utrn*$^{-/-}$ mice. In both models, eosinophils were found to negatively impact the hypertrophic

growth of muscle fibers that occurs during fiber maturation. Further, when eosinophils were continuously depleted from dystrophic mice for a period of 9 weeks following weaning, the average lifespan was significantly extended.

The contribution of eosinophils in muscle repair is not well-understood and is often contradictory in current literature. Discrepancies between the described roles of eosinophils are likely due to differences in eosinophil ablation strategy. Indeed, this has been demonstrated in research examining chronic allergic inflammation (Jacobsen et al., 2014), in which eosinophils are the major contributor to tissue damage (Norman, 1995). The commonly used congenital eosinophil-deficient strains PHIL (Lee et al., 2004) and ΔdblGATA (Humbles et al., 2004) exhibited significant disparities in eosinophil involvement in allergic pulmonary responses, which have since been associated with background strain variability and developmental impacts on other immune cells and tissues arising from the consequence of congenital eosinophil depletion (Jacobsen et al., 2014). Similarly, anti-CCR3 mediated eosinophil depletion has been shown to affect populations of T-lymphocytes and B cells (Gerber et al., 1997; Jinquan et al., 2003). The data presented here, utilizing the inducible eosinophil-deficient iPHIL strain, indicates that eosinophil depletion prior to and during healthy muscle repair improves the robustness of regeneration. This is in opposition with research by Heredia et al., which demonstrates failed muscle regeneration in ΔdblGATA mice (2013). It is possible that this discrepancy is caused by the lifelong loss of eosinophils and background strain variability associated with ΔdblGATA mice.

Eosinophils in dystrophic muscle have largely been found to exhibit cytotoxic effects. In the *mdx* model, eosinophil depletion via anti-CCR3 administration resulted in decreased muscle cell lysis, while genetic ablation of eosinophil-secreted MBP-1 reduced the accumulation of fibrotic tissue in both skeletal muscle and heart (Wehling-henricks et al., 2008). This research also demonstrated an improvement in the pathology of muscular dystrophy following eosinophil depletion. While a difference in the levels of fibrotic tissue were not found, it is possible that this is due to the use of anti-IL-5 as means of eosinophil depletion. IL-5 is an essential mediator in eosinophil activation and differentiation (Clutterbuck et al., 1987); thus, its neutralization does not deplete eosinophils which are already activated and/or differentiated. In addition, while anti-IL-5 has been shown to be capable of depleting nearly 98% of eosinophils in blood (Garlisi et al., 1999), eosinophils which are already present in dystrophic muscle lesions may be less well targeted by anti-IL-5 administration. Alternatively, the 9-week duration of the study may not have been enough time for fibrotic tissue to accumulate to levels in which a statistically significant difference would be present.

In summary, the research presented here describes a novel role for eosinophils in modulating the hypertrophic growth stage of skeletal muscle repair. Eosinophils were found to negatively impact muscle fiber growth and maturation following both acute and chronic injury. Additionally, administration of anti-IL-5 in a severely affected model of muscular dystrophy significantly increased the lifespan. Anti-human IL-5, also known as Mepolizumab, is currently FDA-approved for the treatment of severe eosinophilic asthma, eosinophilic granulomatosis with polyangiitis (EGPA), and hypereosinophilic

syndrome (HES) (Administration; Tantibanchachai, 2020). To our knowledge, this is the first time that anti-IL-5 has been tested for the treatment of DMD and our results demonstrate promising therapeutic potential.

CHAPTER 5

CONCLUSION

Efficient muscle regeneration is dependent upon numerous immune-muscle interactions, therefore understanding the mechanisms that regulate these interactions is paramount for the development of therapeutics aimed to improve muscle regeneration.

In chapter 3, *Mkx* was identified as a transcription factor critical for the initiation of a robust inflammatory response, both in response to acute muscle damage and in response to infection by a bacterial pathogen. In mice lacking *Mkx*, an insufficiency in the number of M1 macrophages at the site of damage lead to a persistence of necrotic myofibers and subsequent impaired muscle regeneration. This deficit in the population of M1 macrophages was also demonstrated *in vitro*, where $Mkx^{-/-}$ BMDM displayed a significant reduction in their capacity to proliferate, as compared to WT. When the immune systems of $Mkx^{-/-}$ mice were challenged with *S. enterica* infection, mice succumbed to infection at lower doses and exhibited a lower LD_{50}. Gene expression analysis in whole muscle at 1- and 3-days post-injury revealed significant down regulation of genes encoding for cytokines and chemokines critical in the inflammatory response following damage.

While analysis of qRT-PCR performed on whole muscle following injury provided valuable information regarding *Mkx* regulation of inflammation, it is difficult to pinpoint the exact cell type(s) that *Mkx* is acting on from this data. Future studies in which specific cell types, such as macrophages, are isolated from injured tissue via fluorescence activated cell sorting (FACS) prior to gene expression analysis would

clarify the mechanism in which the loss of *Mkx* alters the inflammatory response and subsequent muscle regeneration. Using this technique, an investigation into how the expression of *Mkx* changes in specific cell types over the course of repair could also be performed. In the studies presented here, *Mkx* expression was evaluated throughout the course of repair using injured WT tissue (data not shown), however significant expression changes were difficult to measure due to target dilution in whole muscle. Furthermore, an expanded panel of markers to characterize additional cell types that are known contributors to muscle repair, such as FAPs and regulatory T cells, would broaden the understanding of the inflammatory environment present in $Mkx^{-/-}$ injured muscle.

In vitro, MKX has been shown to activate the expression of *Tgfβ* by binding to the *Tgfβ2* promoter during differentiation in MSCs (Huanhuan Liu, Zhang, et al., 2014), while other work has demonstrated TGFβ-induced expression of *Mkx* (Falcon et al., 2019; Perucca Orfei et al., 2019). Thus, it has been hypothesized that *Tgfβ*, released from the ECM following mechanical stress, exposure to proteases, or interactions between integrins and the ECM, participates in a positive feedback loop with *Mkx* to promote remodeling and strengthening of the ECM (Munger & Sheppard, 2011). During skeletal muscle repair, TGFβ is secreted by M2 macrophages and functions to suppress the pro-inflammatory response and promote the production of ECM proteins through inhibition of FAP apoptosis (Lemos et al., 2015). We found significant downregulation of *Tgfβ* in $Mkx^{-/-}$ whole muscle during the first 3 days following injury, which may be in part due to a similar positive feedback loop occurring between *Mkx* and *Tgfβ* in skeletal muscle. Furthermore, production of TGFβ is known to increase following macrophage

phagocytosis of necrotic cells (Fadok et al., 2001; Fadok et al., 1998). The decrease in M1 macrophage numbers and ensuing persistence of necrotic fibers in *Mkx*$^{-/-}$ muscle supports the downregulation of *Tgfβ* expression. Future studies that examine the protein levels of TGFβ in *Mkx*$^{-/-}$ injured muscle would further shed light onto the prospect of an *Mkx*-*Tgfβ* feedback loop in muscle.

One possible mechanism in which *Mkx* could be regulating the recruitment of macrophages to the site of injury may be through eosinophils, which were recently found to display high expression of *Mkx* (de Graaf et al., 2016). Eosinophils have been shown to infiltrate into injured muscle following a time course similar to M1 macrophages (Heredia et al., 2013) and are capable of releasing numerous cytokines and chemokines that are known to modulate other cell types (Jacobsen et al., 2007; Rosenberg et al., 2013). One such example is in white adipose tissue where eosinophil secreted IL-4 was found to be essential for sustaining a population of alternatively activated M2 macrophages that are critical for the maintenance of glucose homeostasis (Wu et al., 2011). When eosinophils were genetically ablated using ΔdblGATA mice, the number of adipose tissue M2 macrophages were greatly reduced. Similarly, eosinophils recruited to the site of muscle damage have been shown to secrete IL-4, which was required for FAPs to proliferate as fibroblasts and aid in debris clearance (Heredia et al., 2013). Confoundingly, the authors found that IL-4 signaling was not necessary for M2 macrophage-mediated muscle repair. This finding is in opposition with other research in which M2 macrophages have been identified as critical contributors in successful muscle repair (Arnold et al., 2007; Ruffell et al., 2009) and thus warrants further examination.

Research performed in the lung examining allergic responses, where eosinophil function has been well-documented, has shown that eosinophils downregulate inflammation and contribute towards repair of damaged tissue (Takeda et al., 2015). Though it was not statistically significant, we did find increased numbers of eosinophils at 2-days post-muscle injury in *Mkx*$^{-/-}$ mice, suggesting the possibility of a heightened presence of eosinophils in the absence of *Mkx*. It is possible that significant differences in eosinophil concentration may be present at earlier (e.g. 1-day post-injury) or later time points (e.g. 3-days post-injury), necessitating future studies in which the peak of eosinophil infiltration is investigated. A heightened eosinophil presence could be responsible for the suppression of the pro-inflammatory response in *Mkx*$^{-/-}$ mice, while also contributing to the necrosis of muscle fibers, as eosinophil secreted MBP-1 has been demonstrated to lyse muscle fibers *in vitro* (Wehling-henricks et al., 2008).

Further support for *Mkx* activity in eosinophils comes from a recent report describing significant upregulation of MKX in pigs experimentally infected with *Trichinella britovi* (Gondek & Herosimczyk, 2020*). T. britovi* is a parasitic nematode whose encapsulated larvae survive within striated muscle and are then transmitted through the consumption of raw meat (Rostami et al., 2017). While the authors are unable to determine the mechanism in which MKX expression is activated during trichinellosis, they postulate that, due to the documented role of MKX in tendon remodeling, MKX is also involved in the remodeling of muscle tissue that occurs in response to nematode infection (Gondek & Herosimczyk, 2020). While this might be possible, it is more likely that the upregulation of MKX stems from the high concentration of MKX-expressing

eosinophils that are associated with parasitic infections. Indeed, eosinophilia following *T. britovi* infection is well documented (Bruschi et al., 2002; Ferraccioli et al., 1988; Fourestie et al., 1993).

In any case, before the function of *Mkx* in eosinophils can be assessed in the context of muscle repair, a more thorough understanding of the role of eosinophils in muscle repair is necessary. Despite the identification of eosinophils in skeletal muscle following an acute injury as well as in the lesions of dystrophic muscle (Cai et al., 2000; Heredia et al., 2013), their overall contribution to muscle repair remains largely unknown. To this end, we first sought to explore eosinophil kinetics during muscle repair using the inducible eosinophil-deficient mouse line, iPHIL (Jacobsen et al., 2014). Eosinophil depletion during the first 7-days following muscle damage led to an increase in regenerating myofiber diameter, suggesting that eosinophils negatively impact myofiber growth and maturation. Similarly, eosinophil depletion in the severely affected muscularly dystrophy mouse model, *mdx*$^{-/-}$*utrn*$^{-/-}$, via administration of anti-IL-5, resulted in increased diaphragm fiber diameters. Perhaps most remarkably, eosinophil depletion also significantly extended the lifespan of *mdx*$^{-/-}$*utrn*$^{-/-}$ mice, which typically die by 20-weeks of age (Deconinck et al., 1997).

With both proposed pro- and anti-inflammatory functions of eosinophils, it is difficult to determine precisely how eosinophil depletion promoted muscle fiber growth. Recent studies in cancer suggest the possibility of different eosinophil subtypes based on the presence of Th1 and Th2 cytokines (Varricchi et al., 2018). This theory has also been supported by *in vitro* experiments in which RNAseq analysis revealed distinct subtypes

of eosinophils following stimulation with either type 1 (IFNγ/TNFα) or type 2 cytokines (IL-33/GM-CSF/IL-4) (Nazaroff et al., 2019). This duality in eosinophil phenotype and function may also be present in healthy muscle repair, akin to M1 and M2 macrophages. Further, a combination of subtypes may be present in dystrophic muscle, as is the case with macrophages (Villalta et al., 2008), muddling the current correlations between eosinophil function in acute vs chronic injury. The recent development of single-cell RNA sequencing allows for the assessment of cellular heterogeneity within whole tissues and thus would be useful in the identification of eosinophil subtypes during the pro- and anti-inflammatory phases of healthy muscle repair, as well as in dystrophic muscle. Indeed, single-cell RNA sequencing has been performed in regenerating muscle, however eosinophils were not specifically identified in these studies (De Micheli et al., 2020; Oprescu et al., 2020). This could be due to several causes, including lack of an enrichment method for eosinophils, low expression of eosinophil-specific transcripts, or grouping of eosinophils with other closely related immune cell types. In addition to shedding light on the gene expression signatures of different eosinophil subtypes, the characterization of other cell types that eosinophils are known to influence, such as macrophages and fibro-adipogenic progenitor cells, would further our understanding of the role of eosinophils in healthy and dystrophic muscle repair. Until markers of eosinophil subtypes are further characterized, future studies in which eosinophils are selectively depleted during the pro- and anti-inflammatory phases of repair, as opposed to throughout both phases of repair, would be necessary in order to assess if eosinophils perform multiple distinct functions during healthy muscle repair.

The characterization of eosinophil subtypes, along with their kinetics, in muscle repair is critical for the possibility of selective depletion of a subtype of eosinophils, which would further enable the ability to fine-tune the inflammatory response in therapeutics used to treat volumetric muscle loss and muscular dystrophies.

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APPENDIX A

PUBLICATION NOTE

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