THESIS

T CELL MEDIATED SATELLITE CELL FUNCTION: IMPLICATIONS FOR AGE-ASSOCIATED CHANGES IN SKELETAL MUSCLE REGENERATION.

Submitted by

Breanna R. Dumke

Department of Health & Exercise Science

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Master's Committee:

Department Head: Richard G. Israel

Advisor: Simon J. Lees

Co-Advisor: Robert W. Gotshall

Melinda A. Frye

ABSTRACT

T CELL MEDIATED SATELLITE CELL FUNCTION: IMPLICATIONS FOR AGE-ASSOCIATED CHANGES IN SKELETAL MUSCLE REGENERATION.

Sarcopenia is an age-associated loss of skeletal muscle mass and strength. Recent evidence suggests that an age-associated loss of muscle precursor cell (MPC) functionality contributes to sarcopenia. Current research also suggests that T cells of the immune system may influence skeletal muscle repair via signaling with MPCs. The objective of the present study was to examine the influence of activated T cells on MPCs. MPCs were collected from the gastrocnemius and plantaris from 3-mo-old (young) and 32-mo-old (old) animals. Splenic T cells were also harvested using anti-CD3 Dynabead isolation. T cells were activated for 48 hours with co-stimulation of 100 IU/ml Interleukin-2 (IL-2) and 5 ug/ml of anti-CD28. Co-stimulation increased 5-bromo-2'-deoxyuridine (BrdU) incorporation (proliferation) of T cells from 13.382% (SEM=4.55, n=5) in control to 64.77% (SEM= 6.02, n=5). Additionally, T cell cytokines increased MPC proliferation by 23.98% (SEM=5.69, n=4) in young MPCs but decreased by 1.58% (SEM=4.09, n=4) in old MPCs. T cell cytokines were also found to be chemoattractant. Young MPCs migrated at a rate of 1.36 (SEM=0.56, n=4) with T cell cytokines. Old MPCs, however, did not migrate with T cell cytokines -0.05 (SEM= 0.214, n=4). These data suggest that T cells may play a critical role in mediating MPC function. Furthermore, aging may alter T cell-induced MPC function. These findings have

implications for developing strategies aimed at increasing MPC proliferation and the regenerative capacity of aged skeletal muscle.

TABLE OF CONTENTS

Chapter I: Introduction

Statement of Problem

Aging is associated with decreased strength and skeletal muscle mass, a condition known as sarcopenia. Contributing factors of sarcopenia include the diminished capacity for aged skeletal muscle to regenerate [1], hypertrophy [2] and re-grow after a bout of atrophy [3]. This decrease in skeletal muscle mass is accompanied by a marked increase in skeletal muscle fibrosis that results in decreased muscle quality. As a result, sarcopenia is strongly associated with decreased quality of life and increased mortality in our aging population [4].

In skeletal muscle, the resident stem cells responsible for tissue repair are termed skeletal muscle satellite cells. Satellite cells were originally named for their anatomical location between the basal lamina and the sarcolemma. In this text, the progeny of activated satellite cells will be referred to as muscle precursor cells (MPCs). Recent evidence suggests that an age-associated loss of satellite cell functionality is the primary factor responsible for the decreased regenerative potential, increased atrophy and increased fibrosis of aged skeletal muscle [5]. MPC function is predominately dictated by the surrounding environment, or the local milieu. However, it is also known that MPCs from aged skeletal muscle respond to the environment differently compared to

MPCs isolated from young muscle [6-14]. One of the main contributing factors to the local milieu of MPCs is infiltrating immune cells.

Following skeletal muscle injury, a rapid, sequential and intricate process of repair that includes initial degeneration then regeneration occurs. During the skeletal muscle repair process, inflammation occurs. The magnitude of the inflammation and regeneration responses directly depends on the severity of the muscle damage [15]. The first stage of the inflammatory response to skeletal muscle injury begins with infiltration of white blood cells (WBCs). Neutrophils, monocytes, and lymphocytes all infiltrate the damaged tissue in a sequential manner. Neutrophils are the first to migrate from the circulation and infiltrate the damaged skeletal muscle. Neutrophils arrive within the injury site immediately following injury and may remain within the skeletal muscle up to 4 days. Neutrophils serve to phagocytose necrotic myofibers and cellular debris during the degeneration phase. They also release pro-inflammatory cytokines such as interleukin (IL)-8 and IL-1, the chemoattractant transforming growth factor- β (TGFβ) and tumor necrosis factor (TNF) [16-22].

Following neutrophil infiltration another type of WBCs, monocytes, migrate to the damaged tissue. Monocyte migration occurs within 1-2 days following injury. Once within the skeletal muscle the monocytes mature and differentiate into macrophages. Macrophages can remain within the skeletal muscle for 7-14 days and are identifiable by their expression of specific cell markers such as ED1+ and ED2+. Both ED1+ and ED2+ macrophages have been shown to influence MPC function and skeletal muscle regeneration. The ED1+ macrophages typically occur in greater concentration in the first 4 days of macrophage infiltration. The appearance of ED2+ macrophages occurs around

day 4 following injury [15, 16, 23-25]. Unlike ED1+ macrophages, ED2+ macrophages are non-phagocytic but instead release growth factors and cytokines critical for regulating satellite cell functions such as fibroblast growth factor-2 (FGF-2), insulin growth factor-1 (IGF-1) and TGFβ [15, 23-25].

Lymphocytes, specifically T cells, also infiltrate skeletal muscle following injury. T cell infiltration occurs within 3 days following injury and T cell concentration remains elevated until 10-15 days post-injury [26, 27]. Inflammatory cytokines such as IL-1 that are present within skeletal muscle injuries may increase T cell activation. Interestingly, activated T cells are able to produce inflammatory cytokines and interferons such as IL-2 and TNF-α that can increase fibroblast production. T cells can also produce anti-inflammatory cytokines such as and IL-4 and IL-10 that are linked to increasing satellite cell migration and the formation of myotubes [26, 27]. Despite the potential for T cells to influence MPC function, current research has yet to examine the role that T cells may play in regulation of skeletal muscle MPC function. The aim of the proposed research was to determine if T cells influence satellite cell migration and proliferation.

Significance of Study

Infiltrating immune cells are thought to be critical mediators of the skeletal muscle repair process. While previous reports have established the importance of macrophages in this capacity, the influence of T cells on skeletal muscle regeneration has yet to be closely examined. As discussed, T cells infiltrate damaged skeletal muscle, are present during regeneration and can release cytokines known to influence MPC function. All together, T cells are likely candidates for MPC regulation. The following

experiments were the first to specifically determine the role of activated T cells on MPC function. Moreover, since aging is associated with impaired skeletal muscle hypertrophy and repair, the influence of age on the communication between T cells and MPCs was investigated.

Specific Aim #1:
Determine the effects of activated T cell cytokine release on MPC function

To examine the influence of activated T cells on MPC function, T cells were isolated from the spleens of young, 3-month-old Fisher 344 X Brown Norway rats and activated via incubation with anti-CD3 and co-stimulation with IL-2 and anti-CD28. T cell activation was verified via 5'-bromo -2'-deoxyuridine (BrdU) incorporation. Conditioned media (CM) from activated T cells was collected and used to treat MPCs. Culture of MPCs with CM provided insight into whether T cells influence satellite cell migration and proliferation; two important aspects of MPC function that are required for skeletal muscle regeneration.

Hypothesis: It was hypothesized that cytokines released from T cells will increase both migration and proliferation of MPCs.

Specific Aim #2:
Determine the effects of age on T cell-mediated MPC function

To determine the effect of age on T cell-mediated MPC function, CM from young T cells was used to treat aged MPCs. T cells were isolated from the spleens of young, 3-month-old, Fisher 344 x Brown Norway rats and activated via incubation with anti-CD3 and co-stimulation with IL-2 and anti-CD28. Satellite cells were isolated from 32-

month-old Fisher 344 x Brown Norway rats. Migration and proliferation of aged MPC was determined following exposure to CM.

Hypothesis: It was hypothesized that MPCs isolated from 32-mo-old animals will exhibit an impaired response to cytokines released from T cells resulting in impaired migration and proliferation, compared to 3-mo-old animals.

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Age-Related Impairment of T Cell Induced Proliferation and Migration of Skeletal Muscle Precursor Cells

[1]Dumke, Breanna R. and [1]Lees, Simon J.

Affiliation:
1-Department of Health and Exercise Science, Colorado State University, Fort Collins, CO

Running Head:
T cells mediate satellite cell function

Corresponding Author:
Simon J. Lees, Ph.D.
Colorado State University
Health and Exercise Science
215F Moby B Complex
Fort Collins, CO 80523-1582
simon.lees@colostate.edu

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Abstract

Sarcopenia is an age-associated loss of skeletal muscle mass and strength. Recent evidence suggests that an age-associated loss of muscle precursor cell (MPC) functionality contributes to sarcopenia. Current research also suggests that T cells of the immune system may influence skeletal muscle repair via signaling with MPCs. The objective of the present study was to examine the influence of activated T cells on MPCs. MPCs were collected from the gastrocnemius and plantaris from 3-mo-old (young) and 32-mo-old (old) animals. Splenic T cells were also harvested using anti-CD3 Dynabead isolation. T cells were activated for 48 hours with co-stimulation of 100 IU/ml Interleukin-2 (IL-2) and 5 ug/ml of anti-CD28. Co-stimulation increased 5-bromo-2'-deoxyuridine (BrdU) incorporation (proliferation) of T cells from 13.382% (SEM=4.55, n=5) in control to 64.77% (SEM= 6.02, n=5). Additionally, T cell cytokines increased MPC proliferation by 23.98% (SEM=5.69, n=4) in young MPCs but decreased by 1.58% (SEM=4.09, n=4) in old MPCs. T cell cytokines were also found to be chemoattractant. Young MPCs migrated at a rate of 1.36 (SEM=0.56, n=4) with T cell cytokines. Old MPCs, however, did not migrate with T cell cytokines -0.05 (SEM= 0.214, n=4). These data suggest that T cells may play a critical role in mediating MPC function. Furthermore, aging may alter T cell-induced MPC function. These findings have implications for developing strategies aimed at increasing MPC proliferation and the regenerative capacity of aged skeletal muscle.

Introduction

Aging is associated with decreased strength and skeletal muscle mass, a condition known as sarcopenia. One contributing factor to sarcopenia is the diminished capacity for aged skeletal muscle to regenerate [1], hypertrophy [2], and re-grow after a bout of atrophy [3]. Moreover, in aging there is a marked increase in skeletal muscle fibrosis in concert with decreased muscle tissue resulting in decreased muscle quality [28, 29]. As a result, sarcopenia is strongly associated with decreased quality of life and increased mortality in our aging population [4].

In skeletal muscle, the resident stem cells responsible for tissue repair are termed satellite cells (originally named for their anatomical location). The progeny of activated satellite cells are referred to as muscle precursor cells (MPCs). Recent evidence suggests that an age-associated loss of MPC functionality is the primary factor responsible for the loss of regenerative potential and increased atrophy and fibrosis of aged skeletal muscle [5]. MPC function is predominately dictated by the surrounding environment, or the local milieu. However, it is also known that MPCs from aged skeletal muscle respond to the environment differently compared to MPCs isolated from young muscle [6-14].

One of the main contributing factors to the local milieu of MPCs is infiltrating immune cells. T cell infiltration into damaged skeletal muscle occurs rapidly following injury [30]. A recent report demonstrated significant T cell infiltration from day 3 until at least day 10 of regeneration [30]. However, very little is known about the specific role of T cells in skeletal muscle repair.

Current evidence suggests that T cells may play an integral role in skeletal muscle repair and fibrosis. Dermatomyositis and polymyositis are disabling rheumatic diseases

characterized by an appreciable number of T cells infiltrating muscle tissue [31]. Moreover, in muscular dystrophy, there is a chronic infiltration of macrophages and T cells due to repeated cycles of degeneration and regeneration [32]. The infiltrating T cells have been implicated in the pathology of muscular dystrophy [32]. Recently, the use of an immunodeficient/dystrophic mouse models demonstrated that T cell depletion resulted in both reduced transforming growth factor-β1 (TGF-β1) and skeletal muscle fibrosis in dystrophic skeletal muscle [33, 34]. These immunodeficient/dystrophic mice demonstrated improved skeletal muscle regeneration and decreased fibrosis [33, 34].

Thus, one possible interpretation is that the chronic infiltration of T cells in the lesions of dystrophic skeletal muscle promotes the fibrotic aspects of muscular dystrophy. The role of T cells in skeletal muscle repair of non-dystrophic skeletal muscle is not known. Transient T cell infiltration after an acute bout of injury in non-dystrophic muscle may provoke a different outcome on the regenerative process, compared to the chronic infiltration observed in dystrophic skeletal muscle. Moreover, it is known that non-dystrophic animals lacking T cells (nude mice) exhibit impaired muscle growth [35], indicating a possible role for T cells in myogenesis.

T cell infiltration begins at the early stages of regeneration during MPC migration and proliferation, suggesting that T cells may play an integral role in skeletal muscle repair. Despite the simultaneous accumulation of T cells and MPC action, very little is known about the potential regulatory function of T cells on MPCs. However, in terms of sarcopenia, delineating mechanisms of T cell-mediated MPC function in aging may facilitate the development of treatment strategies aimed at increasing skeletal muscle mass and strength in our aging and physically frail populations.

The purpose of the present study was two-fold. The first was to establish whether activated T cells regulate MPC proliferation and migration. In order to do this, MPCs isolated from young rats were treated with conditioned media collected from activated T cells. The second was to establish whether an age-related change exists in the responsiveness of MPCs to activated T cells. In order to do this, MPCs were isolated from skeletal muscle of aged/sarcopenic rats and T cell-mediated proliferation and migration were determined.

Materials and Methods

Animals

All procedures were approved by the Institutional Animal Care and Use Committee at Colorado State University. 3-month-old (3-mo) and 32-month-old (32-mo), Fisher 344 x Brown Norway F1 hybrid, male rats were obtained from the National Institute on Aging. Animals will be housed at 21°C on a 12-hr light/12-hr dark cycle and allowed free access to food and water. At the time of sacrifice, animals were given an intraperitoneal injection of ketamine (80 mg/kg), xylazine (10 mg/kg), and acepromazine (4 mg/kg) and the tissue was excised.

MPC Isolation and Culture

MPC isolation was modified from Allen et al. [36] as described previously [8, 13]. Briefly, cells were isolated from the gastrocnemius and plantaris muscles by pronase digestion and pre-plated for 24 hours on tissue-culture treated 150-mm plates. MPCs were cultured on Matrigel (BD Biosciences, San Jose, CA) coated plates (0.1 mg/ml Matrigel, 60 minutes at 37°C) and passaged only one time (growth media, 20% FBS in Ham's F-10; 6% O_2, 5% CO_2, and 89% N_2 at 37° C) (Figure 1).

T Cell Isolation and Culture

Splenocytes collected from the spleen were incubated with the mouse monoclonal antibody to rat CD3 (CALTAG Laboratories, Invitrogen, Carlsbad, California) followed by incubation with anti-mouse IgM Dynabeads (Dynal, Invitrogen, Oslo, Norway). CD3 cells were then separated using the Dynabead magnet and re-suspended in 10ml OpTmizer T-cell Expansion media (Gibco, Invitrogen, Carlsbad, California). T cell stimulation was induced with treatment of 100 IU/ml of IL-2 (PreproTech, Rocky Hill, New Jersey) and 5μg/ml of anti-CD28 (BD, San Diego, California). After 2 days, cells were either collected to assess activation/proliferation or the media was changed to a basal media (BM) for cytokine collection. Cells used for activation/proliferation assessment were pulsed with 5-bromo-2'-deoxyuridine-5'-monophosphate (BrdU) and fixed for analysis via flow cytometry. For cytokine collection, following the two-day T cell stimulation, the media was changed to BM (2% FBS in DMEM) and cells were cultured for an additional 24 hours. During this time, the T cells released cytokines into the cell culture media, a process known as conditioning media (Figure 2).

MPC Migration

Following the first passage, MPC migration was measured using the FluoroBlok cell migration plates (8 μm pore size; BD Biosciences, San Jose, CA) with inserts coated with Matrigel. 75,000 MPCs were loaded into the upper chamber of the migration plate in BM containing calcein-AM (5 μg/ml). The lower chamber contained either BM, or conditioned media (CM) obtained from T cells. To detect the rate of migration, the plates were read with bottom reading fluorescence (Ex485/Em530) every 30 minutes up to 120 minutes (Soft Max Pro Software; Spectra Max M5, Molecular Devices, Silicone Valley, CA) (Figure 1).

Proliferation

To analyze cell proliferation of both T cells and MPCs, BrdU incorporation was determined using flow cytometry [37]. As mentioned above, T cells were pulsed with BrdU for 60 min beginning 47 hours following isolation in either the presence or absence of IL-2 (100 IU/ml) and anti-CD28 antibody (5 μg/ml). For MPC proliferation, cells were plated in 10% FBS in Ham's F-10 and cultured for 24 hours. After 24 hours, the media was replaced with a 1:1 mixture containing one part 20% FBS in Ham's F-10 and either BM or CM. MPCs were then pulsed with BrdU for 60 min beginning 23 hours following treatment with either BM or CM. Briefly, after the 60 min pulse, the cells were washed twice with PBS, removed from the plates, centrifuged at 500 x g for 5 min, and fixed with ice-cold 70% ethanol. DNA was acid-denatured (2 N HCl, 30 min) and incorporated BrdU was detected using a fluorescein-conjugated monoclonal antibody raised against BrdU (5 μg/ml, Roche Applied Sciences, Indianapolis, IN) in PBS with 0.1% bovine serum albumin (BSA). 20,000 cells were analyzed using a Epics XL-MCL Coulter flow cytometer (Beckman Coulter, Brea, CA) and FCS Express (De Novo Software, Los Angeles, CA) (Figure 1) [8].

Statistics

Data are presented as mean ± SE. Sample sizes are indicated for each measurement in the figure legends, where n represents independent isolations from separate animals. Comparisons between groups were done using the two-way repeated measures ANOVA (SigmaStat software, Systat, Chicago, IL). Significance was accepted at $p \leq 0.05$.

Results

Since T cells infiltrate damaged skeletal muscle [26, 30] within the same time period of MPC proliferation, migration, and differentiation, T cells may regulate MPC function [15, 30, 33, 38]. However, despite this link, very little is known regarding the influence of T cells on MPC function.

In order to determine if T cells may contribute to MPC function naïve splenic T cells were isolated and activated *in vitro*. Following the isolation of CD3 T cells from the spleen, T cells were activated via co-stimulation with IL-2 (100 IU/ml) and anti-CD28 antibody (5 μg/ml) (Figure 3A). In order to verify successful activation of the T cells via co-stimulation proliferation was determined using BrdU incorporation. Based on the flow cytometry analysis (Figure 3B), the combined IL-2 and anti-CD28 stimulation significantly increased T cell proliferation compared to isolated CD3 T cells that did not receive the combined IL-2 and anti-CD28 treatment. T cell stimulation increased T cell proliferation by almost 5-fold. T cells that were co-stimulated were 64.8% BrdU positive, while only 13.4% of T cells that did not receive combined IL-2 and anti-CD28 treatment were BrdU positive.

In order to determine the potential effect of T cell-released cytokines on MPC function, activated T cells cultures were changed to BM and allowed to condition the media for 24 hours. CM was then collected and used to treat MPCs prior to pulsing with BrdU. First, proliferation of MPCs isolated from 3-mo and 32-mo animals was determined in media containing the BM (1:1 mixture of 20% FBS in Ham's F-10 and BM). Under these conditions, there was no difference in the proliferation of MPCs isolated from either age (Figure 4a).

However, when MPCs were treated with CM, age-related differences emerged. CM from activated T cells caused an increase in proliferation of MPCs isolated from 3-mo animals (Figure 4b). However, CM had no effect on the proliferation of MPCs isolated from 32-mo animals (Figure 4b). These data indicated that MPCs isolated from old skeletal muscle are not responsive to the mitogenic factors released from activated T cells.

Following skeletal muscle injury, MPCs migrate to the site of injury. The responsiveness to chemotactic factors that attract MPCs to the site of injury is a critical step in the regenerative process. In order to determine if age-associated differences exist in the MPC response to chemotactic agents, a migration assay was performed. The migration of MPCs isolated from 3-mo and 32-mo were first tested in BM. Migration towards BM was tested and the appearance of migrated cells was measured every 30 minutes for 2 hours (Figure 5A). A higher number of MPCs isolated from the 3-mo-old animals had migrated at the 90 and 120 minute time points compared to MPCs isolated from the 32-mo-old rats (Figure 5A). The slope calculated from the regression line plotted for the cell migration indicates the relative rate of migration of MPCs. From the slopes it was determined that the MPCs isolated from young animals exhibited a higher rate of migration, compared to MPCs isolated from aged animals (Figure 5B). These data indicate that there may be an intrinsic deficiency in MPC migration with age.

Activated T cells are known to release chemokines. In order to determine if activated T cells promote MPC chemotaxis, cell migration towards CM was performed. CM-induced migration of MPCs was examined at 30-minute intervals for 2 hours. A higher number of MPCs isolated from the 3-mo-old animals had migrated at the 60, 90,

and 120 minute time points compared to MPCs isolated from the 32-mo-old rats (Figure 6A).

However, since migration occurs in BM alone, in order to determine the cell migration induced by the chemokines in CM, migration in BM was subtracted to give T cell-induced migration. In response to T cell released chemokines, more MPCs isolated from the 3-mo-old animals had migrated at the 60, 90, and 120 minute time points compared to MPCs isolated from the 32-mo rats (Figure 6B). Based on the slopes, MPCs isolated from young animals exhibited a higher rate of migration towards T cell released factors, compared to MPCs isolated from aged animals (Figure 6C). Importantly, CM induced a significant increase in the rate of migration of MPCs isolated from 3-mo animals, while there was no difference in rate of migration of 32-mo animals in towards CM or BM. These data indicate that MPCs isolated from old skeletal muscle do not respond to the chemotactic agents released from activated T cells.

Discussion

Aging is associated with diminished MPC function, leading to impaired skeletal muscle regeneration [5]. Infiltrating immune cells are a main contributor to the local milieu of MPCs in injured skeletal muscle. Despite the known importance of the infiltrating immune cell on the regenerative process [39], very little is known about a potential regulatory role of T cells influencing MPC function. To our knowledge, we are the first to demonstrate that cytokines released from activated T cells possess both mitogenic and chemoattractant functions on MPCs isolated from young skeletal muscle. Moreover, MPCs isolated from aged skeletal muscle are not responsive to the mitogens and chemokines released from activated T cells. Taken together, our findings support the

notion that intrinsic differences exist between MPCs isolated from skeletal muscle of young and old animals. The inability of aged MPCs to respond to environment cues of infiltrating immune cells may contribute to age-associated impaired skeletal muscle regeneration.

A link exists between inflammatory cell function and the skeletal muscle repair. When non-steroidal anti-inflammatory drugs (NSAID) are administered, there is impaired muscle regeneration [40, 41] that leads to weaker musculotendinosus units compared to untreated animals [42, 43]. It is also been established that NSAID treatment and specific inhibition of cyclooxygenase enzymes causes diminished MPC function [44, 45]. More recently, macrophage depletion led to increased fibrosis in regenerating skeletal muscle post-injury [46].

It is known that infiltrating cells of the immune system into damaged skeletal muscle are part of a well-choreographed set of events during skeletal muscle repair. Following skeletal muscle injury, white blood cells (WBCs) infiltrate into the skeletal muscle compartment [22, 47-51]. Neutrophils are the first to migrate and arrive within the injury site immediately following injury peaking between 1 to 4 hours post-injury and may remain within the skeletal muscle up to four days [52-54]. Neutrophils serve to phagocytose necrotic myofibers and cellular damage. Neutrophils also release pro-inflammatory cytokines such as IL-6, TGF-β and TNF-α that may augment the inflammatory process [52, 54-59]. Additionally, neutrophils release IL-1 and IL-8 that are chemoattractant cytokines for macrophages [22].

Within 24 hours, the concentration of neutrophils within the damaged muscle decreases [26] and monocyte infiltration begins. Once within the skeletal muscle the

monocytes mature and differentiate into tissue macrophages. Macrophages can remain within the skeletal muscle for 7-14 days. The ED1+ macrophages typically occur in greater concentration in the first four days of macrophage infiltration. ED1+ macrophages phagocytose damaged myofibril debris and release pro-inflammatory cytokines such as IL-1, IL-2, interferon-γ (IFNγ) and tumor necrosis factor α (TNFα) [22, 54, 60-63]. The appearance of ED2+ macrophages occurs around day four. ED2+ macrophages are non-phagocytic but instead release growth factors and cytokines critical for regulating muscle precursor cell functions. ED2+ macrophages release anti-inflammatory cytokines including IL-4, IL-5, IL-6, IL-10 and IL-13 and are thought to promote tissue repair [22, 25, 64-66].

While less studied than macrophages, T cell infiltration also occurs following skeletal muscle injury. A recent report examining post-injury immune cell infiltration of T cells was similar to macrophages at day 3 and remained significantly elevated until at least day 10 of regeneration [30]. Importantly, the time course of T cell infiltration overlaps with the regenerative phase following skeletal muscle injury. The current findings support a possible role for T cells in mediating MPC function leading to tissue repair. Since MPC migration begins within the first 24 hours [67] and proliferation is first detected after the first day following injury [68], early mitogenic/chemoattractant cues are likely to come from alternate sources (e.g., neutrophils). However, T cell-released chemokines may serve to retain MPCs at the site of injury/repair and the pro-mitogenic function of T cell-released cytokines may play a role in continued proliferation of MPCs at the site of injury. The data collected in the present study support the notion that T cells are able to stimulate MPC function during skeletal muscle regeneration.

Aging is associated with decreased strength and skeletal muscle mass, a condition known as sarcopenia. Sarcopenia leads to decreased quality of life and increased mortality in our aging population. One contributing factor to sarcopenia is the diminished capacity for aged skeletal muscle to regenerate [2], hypertrophy [69] and regrow after a bout of atrophy [3]. In addition, there is a marked increase in skeletal muscle fibrosis in concert with decreased muscle tissue with aging. Recent evidence suggests that an age-associated loss of MPC functionality is the primary factor responsible for the loss of regenerative potential and increased atrophy and fibrosis of aged skeletal muscle [5]. While MPCs have a clearly defined role in facilitating skeletal muscle regeneration, MPCs isolated from aged skeletal muscle have been shown to exhibit a transition from a myogenic to a fibrogenic lineage [69, 70]. Also, previous reports that demonstrate that aged MPCs respond differently to environmental cues compared to MPCs isolated from young muscle [7-14]. In the present study we demonstrate that MPCs isolated from aged skeletal muscle fail to respond to the mitogenic agents released from activated T cells. In addition, MPCs isolated from aged skeletal muscle exhibited diminished migration (~40% slower rate of migration towards BM). More importantly, while chemokines from activated T cells increased the rate of migration MPCs isolated from young skeletal muscle ~2-fold, MPCs isolated from aged skeletal muscle did not respond to these chemoattractant agents.

A better understanding of the potential role immune cells may exert on MPCs is vital to delineating mechanisms responsible for skeletal muscle regeneration. Just as important, the age-associated differences in the response to environmental cues driving

MPC function need to be identified. To our knowledge, this study was the first to specifically examine influence of T cells on MPC function.

In this report we have established that activated T cells have the capacity to promote both proliferation and migration of MPCs isolated from young skeletal muscle. However, MPCs isolated from aged skeletal muscle are not responsive to the mitogenic factors released from activated T cells. Moreover, MPCs isolated from aged skeletal muscle did not respond to the chemoattractant agents released from activated T cells. Taken together, these data suggest that T cells may mediate MPC function during skeletal muscle regeneration and aging may alter T cell-induced MPC function. While future studies are needed to identify specific influences of T cells on MPC function in aging, these findings have implications for the development of potential treatment interventions aimed at improving the skeletal muscle repair process as we age.

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Chapter III: Review of the Literature

Mammalian Skeletal Muscle

Structure

Skeletal muscle is a critical organ for all mammals and is primarily responsible for locomotion, posture and breathing [71]. At the simplistic level, skeletal muscle is primarily composed of two main components: myofibers and connective tissue [72]. The myofibers are responsible for skeletal muscle contraction while the connective tissue provides the supportive framework that connects all myofibers within a muscle together. Skeletal muscles are also highly vascularized and contain nerves. Blood vessels provide nutrients and remove waste while motor neurons transmit afferent signals to initiate skeletal muscle contractions [71]. Skeletal myofibers vary in length and size. Within humans, myofibers may only be a few millimeters long as in the stapedius muscle of the inner ear or up to 50 millimeters long within the sartorius muscle of the leg. The diameter of muscle fibers also varies between 15 to 100 mm depending on muscle function and myofiber arrangement such as parallel versus pennate fibers [72].

Within each myofiber, there are repeating contractile units of proteins known as sarcomeres. Sarcomeres are organized structures of contractile proteins known as myosin and actin. The basic mechanism of a muscle contraction is similar within all myofibers types and is the result of a myosin-rich thick filament “sliding” over the actin-rich thin filaments after initiation by a skeletal muscle motor neuron [71, 73].

As a whole, skeletal muscle is composed of numerous, long, cylindrical myofibers that are individually surrounded by a thin connective tissue layer called the endomysium. These parallel muscle fibers are grouped into small bundles and enclosed by the perimysium. Together grouped muscle fibers are known as fascicles. Groups of fascicles are bound together and surrounded by the epimysium to form the entire muscle (Figure 7). All of the connective tissues within muscle are continuous connective tissues that all attach to tendons at both the origin and the insertion of the muscle [42].

Injury & Repair

Trauma to skeletal muscle can be of severe magnitude, macrotrauma, or of lesser magnitude, microtrauma [43]. Muscle injuries are mainly the result of contusions, strains, lacerations and age related injuries. Within sports, more than 90% of muscle injuries are contusions and strains. Muscle contusions occur when a muscle is damaged by a sudden, forceful compression such as a direct hit to the muscle. Strains are caused by excessive tensile force that overstrain the muscle and may cause ruptures within the muscle. Most strain ruptures occur at the myotendinous junction (MTJ). Each skeletal muscle is attached at both ends to the connective tissue of a tendon or tendon-like fascia at a MTJ. Typically, muscle strains occur within superficial muscles that span two joints such as the rectus femorus or gastrocnemius [42, 72].

When skeletal muscle is damaged, an intricate and extensive repair process involving several cellular responses heals the tissue. There are two phases that occur within skeletal muscle repair: the degeneration phase and the regeneration phase [71]. The degenerative phase occurs immediately following injury while the regenerative phase begins as early as 24 hours following injury and lasts for several days. Depending on the injury, overlap can occur between degeneration and regeneration processes. Although

almost all muscle repair mechanisms undergo the same sequential series of degeneration and regeneration phases, the extent and duration of each phase is in proportion to the muscle injury severity [71, 74-77]. The rate of muscle repair is also influenced by other factors such as level of physical activity prior to and post-injury, immobilization and age [42].

Degeneration

During the destruction phase, the initial skeletal muscle injury and necrosis of the myofibers occurs. This is event is generally triggered by the disruption of skeletal muscle sarcolemma that exposes the inner cell structure contraction bands and increases myofiber permeability. Following initial injury, normal sarcomeric organization is lost and myofibrils retract from the injury site. This creates a retraction region where the sarcolemma and endomysium proteins are left surrounding the damaged area of the muscle fiber [78]. Fortunately, contractions band at the Z-disc between sarcomeres serve as barriers that seal off the damaged tissue and prevent the muscle necrosis at the site of injury from spreading the entire length of the skeletal muscle [72]. However, due to the destroyed myofiber integrity, creatine kinase leaves the sarcoplasm and intramuscular calcium homeostasis is lost. Through these processes, calpains and calcium-activated proteases are activated and cleave damaged myofibrilar and cytoskeletal proteins resulting in either focal or total autolysis of myofibers [71, 79, 80].

The degeneration phase is also accompanied by activation of mononucleated inflammatory cells. When skeletal muscle is injured, the blood vessels within the skeletal muscle are damaged as well. Damaged vasculature facilitates the infiltration of circulating inflammatory cells to the injury. Current research is beginning to report that factors released by damaged skeletal muscle may activate inflammatory cells such as

neutrophils, macrophages and T cells which then contribute to removal of cellular debris and the regeneration of skeletal muscle through the promotion of MPC function [78, 81]. The detailed roles of immune cells with skeletal muscle injury are discussed later.

Repair

Following skeletal muscle degeneration, regeneration begins. During the regeneration phase, repair of damaged connective tissue and muscle fibers occurs. Reinnervation as well as revascularization also occurs during the regeneration phase. During skeletal muscle injury, platelets and additional fibrillar proteins such as fibrin infiltrate the damaged tissue. Both fibrin and fibronectin form cross-links to create an initial extracellular matrix (ECM) that serves as a scaffold and anchorage site for invading fibroblasts. Fibroblasts synthesize proteins and proteoglycans such as tenascin-C (TN-C) and additional fibronectin to also build the ECM framework. Both TN-C and fibronectin have elastic properties that enhance the contractile strength of the skeletal muscle. The newly formed ECM also provides initial strength to the damage skeletal muscle so that it can withstand any normal contraction forces. [72].

While the initial connective tissue scar at the injury site is the weakest point within the injured skeletal muscle, the tensile strength increases with the production of type I collagen. Type I collagen is produced after fibronectin as part of the remodeling process. Additionally, within ten days post-trauma the scar matures to become one of the strongest points within the skeletal muscle. If a skeletal muscle is loaded to failure after complete repair, a new skeletal muscle rupture would occur adjacent to the newly formed MTJs of the regenerated myofibers and the scar tissue. However, while most skeletal muscle injuries will heal without a functionally disabling fibrous scar, sometimes the scar tissue can be excessive [72].

In addition to the connective tissue repair, skeletal muscle repair occurs. Skeletal muscle regeneration is an incredible process requiring layers of intricate cellular processes and a critically important group of myogenic stem cells named satellite cells [46].

Skeletal Muscle Stem Cells

Historical Background

Despite muscle fibers being post-mitotic, mammalian adult skeletal muscle still exhibits a remarkable ability to respond to physiological demands such as growth, training and injury. Skeletal muscle growth and regeneration is attributed to a resident population of adult stem cells known as skeletal muscle satellite cells. Satellite cells were first identified within a frog myofiber by electron microscopy in 1961 by Mauro [82]. The cells were described to be mononucleated cells positioned between the inner sarcolemma of a muscle fiber and the basal membrane that wraps around the whole muscle fiber. Due to their adjacent position to muscle fibers, the cells were termed satellite cells (Figure 8) [82].

To extend the initial finding by Mauro [82], Muir, et al. [47] examined the skeletal muscle from fruit bats and mice to confirm the presence of satellite cells. Similar to Mauro's findings, satellite cells were found outside the sarcoplasm and contained centrioles, Golgi apparatus and mitochondria. However, Muir noted the satellite cells did not contain myofilaments [47].

Function

During development, skeletal muscle myogenesis begins with somatic mesoderm derived multipotential mesodermal cells [71, 83]. Depending on signaling from

surrounding tissues, the multipotential mesodermal will commit to a myogenic lineage due to up-regulation of myogenic regulator factor family (MRF) transcription activators, MyoD and Myf5. These MyoD and Myf5 positive cells then proliferate into myoblasts and eventually fuse together to form multinucleated myogenic cells that mature into contracting skeletal muscles. During muscle development, another subpopulation of myoblasts fails to differentiate and remains quiescent. These myoblasts remain associated with the surfaces of developing myofibers and become skeletal muscle satellite cells [71]. After natal growth, skeletal muscle fibers are post-mitotic and cannot independently undergo cellular division and growth. Instead mature skeletal muscle fibers rely on satellite cells for hypertrophy, repair and regeneration [48, 71].

In adult muscle, satellite cells are quiescent, undifferentiated, mononucleated cells. Satellite cells compose about 2-7% of the nuclei associated with a single muscle fiber (Figure 9) [49, 50]. When skeletal muscle myotrauma from exercise or injury occurs, satellite cells within the damaged fibers activate and proliferate [67]. Typically activation of satellite cells from adjacent muscles does not occur but can if damage is extensive and spans multiple fibers [67, 84]. The time at which satellite cell activation and proliferation occurs varies depending on the injury but can occur as early as 24 hours post-injury [85-87].

At the molecular level, when quiescent satellite cells activate, rapid up-regulation of two MRFs, Myf5 and MyoD, occurs. During satellite cell activation, MyoD up-regulation appears to be the earliest signal of activation and is detectable before any signs of proliferation [68, 88-97]. Once activated, the satellite cells proliferate. In this review, the progeny of activated satellite cells are referred to as muscle precursor cells (MPCs).

When satellite cells proliferate, some of the MPCs return to a quiescent state to regenerate the satellite cell pool. Comparatively, some of the new MPCs migrate to the site of injury and fuse with preexisting skeletal muscle to regenerate the damage skeletal muscle. Depending on the severity of the injury, MPCs can align and fuse together to form new myofibers (Figure 10) [51].

Once at the injury site, myogenin and MRF4/herculin/myf-6 (one gene that has three names from three different laboratories) are upregulated to begin MPC differentiation and fusion to the damaged skeletal muscle tissue [89-93]. In addition to myogenin and MRF4, MyoD [98] has also emerged to be a vital regulator of differentiation [53]. These genes are transcription factors that promote the up-regulation of additional transcription factors and overall control myogenic lineage determination [52, 53, 55-57].

To examine the time course of MPC gene expression during skeletal muscle regeneration, Paoni, et al. [58] conducted acute hind limb ischemia in young mice using femoral artery ligation (FAL). During this study, Paoni, et al. examined muscle function, histopathology, and gene expression using DNA microarrays at several time points ranging from four hours to 28 days post-FAL. Following FAL injury, MPC differentiation can begin as early as 24 hours post-injury as noted by the induction of MyoD expression. Within 3 days post-FAL, gene expression of MPC transcription factors and myogenic proteins peaked indicating a rapid regeneration process by MPCs. At 7 days post-FAL, full skeletal muscle regeneration was completed. This was noted by peaked muscle contractile protein expression and the return of normal muscle function by the seventh day. From this study, it is found that MPCs rapidly begin to aid in skeletal

muscle regeneration as early as 24 hours post-injury and reach peak regeneration to complete skeletal muscle repair within 7 days following injury [58].

To examine the importance of skeletal muscle satellite cells in hypertrophy, Adams, et al. [58] used gamma irradiation of rat skeletal muscle prior to skeletal muscle overload. Gamma irradiation is a technique used to ablate satellite cell function and impair regeneration [58, 99, 100]. Gamma irradiation was performed on the left hind limb of young rats and the gastrocnemius and soleus muscles were removed to functionally overload the plantaris muscle. Tissue collection was performed at and 6 hours, 24 hours, 3, 7, 15, and 90 days following the procedure. Despite functional overload, the skeletal muscle irradiated did not develop compensatory hypertrophy [58].

From Adams, et al. [58] support of myonuclear domain is provided. During hypertrophy, skeletal muscle increases in myofibrillar protein content, cross-sectional area, and mass. Since each myofiber is multinucleated, each nucleus has a nuclear domain. A nuclear domain is the cytoplasm volume within a myofiber that is regulated by the gene products of one myonucleus. Within skeletal muscle, myonuclear domain (the ratio between myonuclei and cytoplasm volume) is kept relatively constant. As a result, the addition of myonuclei is required in order to increase myofiber volume. Since adult skeletal muscle is post-mitotic, satellite cells and their MPC progeny are required to add new myonuclei to the preexisting skeletal muscle during hypertrophy [101]. Thus, in the Adams, et al. [58] study compensatory hypertrophy was impaired due to the ablation of satellite cells.

It is also important to note that with small increments in myofiber cross-sectional area and cytoplasmic, volume preexisting myonuclei transcriptional and translational

activity can be sufficient [101]. During early periods of hypertrophy, some studies have found satellite cell-independent myofiber growth [102-104]. However, there is a threshold at which additional increases in myofiber size requires additional myonuclei as supported by the Adams, et al. study [58] and other studies [99, 100].

Skeletal Muscle [Ion] & MPC Function

In skeletal muscle, ion concentrations and membrane potentials can influence satellite cell and MPC functions. Resting, quiescent satellite cell membrane potential is -31± 16mV whereas surrounding muscle fibers have a resting potential of -60mV [105, 106]. While resting membrane potentials are highly regulated, the membrane potential of both can change depending on stimuli.

Post-contraction, the intracellular concentration of ATP within skeletal muscle decreases. As a result, the ATP sensitive K^+ channels open that eventually decrease the activity of the Na^+/K^+ membrane pumps. As a result, the intracellular sodium concentration increases as the extracellular potassium concentration increases, leading to a reduction in the transmembrane potential. Essentially, high transmembrane potential levels are indicative of an increased $[K^+]$ to $[Na^+]$ ratio.

Once activated, proliferating satellite cells have low resting potentials around 8 mV and during differentiation and myotube development MPC resting potentials increase to about -60mV [107]. Interestingly, a low transmembrane potential is known to aid initiation of proliferation and increase DNA synthesis. Comparatively, high transmembrane potentials can block mitosis of MPCs. It has been shown that satellite cells undergo an influx in Na^+ prior to the commencement of proliferation and during proliferation. Additionally it has been found that external $[Na^+]$ can stimulate cell proliferation in a dose dependent manner. From in vitro studies, maximum MPC

proliferation has been reported to occur in media at a concentration of 100mM NaCl. Half-maximum proliferation occurs at 60-70mM NaCl and cells are no longer induced to proliferate at concentrations of ≤20mM NaCl [108]. With injury, if skeletal muscle membrane integrity is lost and the interstitial concentration of Na^+ increase, potentially this may help activate and increase satellite cell proliferation.

External Mitogen Regulation

To gain more insight into MPC functions and regulation, current research is starting to examine how MPC activity is controlled. Presently, it is thought that MPC function is controlled through both external cues and the internal response to environmental cues (Figure 12). Currently, much research has focused on identifying and examining the influence of growth factors, cytokines and interleukins on MPC chemotaxis, proliferation and differentiation. One of the first studies that began the examination of the regulation of MPC function was by Bischoff in 1986. In this study, it was found that MPCs cultured on single viable fibers could be activated and proliferated in response to crushed skeletal muscle extract (CME). This observation suggested that damaged skeletal muscle fibers release mitogens that induce MPC proliferation [59].

Once Bischoff discovered that CME is a potent mitogen for cultured satellite cells, much effort has focused on identifying different cytokines, chemokines and molecular molecules that regulate satellite cell function. Currently, mitogens including fibroblast growth factor (FGF), hepatocyte growth factor (HGF), insulin-like growth factor I (IGF-I), insulin-like growth factor II (IGF-II), transforming growth factor (TGF), leukemia inhibitory factor (LIF), interleukin-6 (IL-6), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), transferrin (Tf), adrenocorticotrophin (ACTC),

and macrophage colony stimulating factor (M-CSF) have been found to influence MPC function [48].

Fibroblast Growth Factor

FGFs are a family of cytokines that have nine different isoforms (FGF-1 to FGF-9) [48]. Out of all of the isoforms FGF-6 and FGF-2 are of particular interest with MPC regulation. During the skeletal muscle regeneration phase, FGF-6 expression is upregulated in muscle. Additionally, the transcription of FGF receptor (FGF-R) also increases fivefold and further increases when HGF is present [48, 54]. Through many studies, FGF-6 and FGF-2 have both been shown to be potent stimulators of satellite cell activation and proliferation [54, 60, 61]. For example, proliferation of MPCs cultured on a single muscle fiber increased in a positive dose dependent response to FGF-6 [62]. Additionally, FGF-6 genetic knockout mice have impaired satellite cell proliferation and skeletal muscle regeneration following crush injury [58].

The FGF cytokines are also potent inhibitors of differentiation. Currently, FGFs are thought to block an early stage of terminal myogenic differentiation. However, the specific mechanism by which FGFs regulate myoblast function is unknown. Potentially FGFs may inhibit expression of MyoD1 and myogenin, two genes critical for MPC differentiation [48, 54].

Hepatocyte Growth Factor

HGF was originally associated with skeletal muscle regeneration by Jennishe, et al. [109] who found HGF within regenerating muscle. Now it is commonly accepted that HGF is a multifunctional cytokine expressed within satellite cells and myofibers along with its receptor c-Met. Both HGF and c-Met expression increase in proportion to the severity of muscle injury. When skeletal muscle is damaged, HGF bound to the fiber

surface is released and can bind to the high-affinity receptor c-met expressed on both quiescent and activated satellite cells [63]. Through several studies, HGF has been found to induce quiescent satellite cell activation [109] and promote satellite cell activation [65, 110, 111]. HGF has been shown to be a potential chemotactic and activator for MPCs [64, 65]. HGF is also an inhibitor of differentiation [48, 54, 66].

Insulin-like Growth Factor –I and -II

In addition to regulation of insulin metabolism, IGF-I and IGF-II are important for satellite cell proliferation and differentiation. IGFs are a family of small peptides that are structurally similar to pro-insulin [48]. Both IGF-I and IGF-II are upregulated during skeletal muscle regeneration [112, 113] and promote both proliferation and differentiation/fusion of myoblasts through altering myogenic regulatory factors (MRFs) [36, 114-118].

Additionally, IGF-I has been shown to enhance satellite cell proliferation and muscle fiber survival through multiple signaling pathways such as calcineurin/NFAT, mitogen-activated protein (MAP) kinase, and phosphatidylinositol-3-OH (PI-3K) pathways [71, 119]. IGF-I also increases satellite cell differentiation through the PI-3K pathway and Akt activation [119, 120]. IGFs may also trigger beneficial processes for MPC differentiation such as amino acid uptake and protein incorporation, uridine and thymidine incorporation into nucleic acids, glucose uptake, and cell proliferation. These processes are important for MPC differentiation since they aid in protein synthesis and muscle fiber repair [3, 48, 116, 121-123]. Additionally, IGFs may promote skeletal muscle regeneration through motor neuron reinnervation [124, 125].

Transforming Growth Factor-β

TGF-β is a member of a protein super family that includes multiple TGF-β forms,

activins, inhibins and other proteins. TGF-β has been shown to be a potent inhibitor of myoblast differentiation. Generally, TGF-β silences the transcription activation of the MyoD proteins that inhibit muscle proliferation and differentiation. In the presence of IGF-I or FGF, TGF- β cannot suppress proliferation but can still inhibit differentiation [48, 117, 126, 127].

Leukemia Inhibitory Factor & Interleukin-6

LIF and IL-6 are members of the same IL-6 cytokine family and are produced by multiple cells including MPCs and macrophages. Both of these cytokines bind to the same receptor and generate similar actions. LIF has been shown to increase skeletal muscle regeneration while IL-6 promotes necrotic tissue degradation and satellite cell proliferation [48].

In addition to growth factors that regulate MPC function, cytokines and interleukins released from immune cells are becoming a focus by researchers. From current research, the importance of the immune system with skeletal muscle repair has emerged. As mentioned, following injury, skeletal muscle fibers undergo degeneration and an inflammatory reaction occurs. During the inflammatory process, immune cells including leukocytes, monocytes/macrophages, and T and B cells infiltrate the damaged tissue. This is followed by satellite cell activation. Within a few days post-injury, satellite cells within the muscle have migrated to the site of injury, proliferated, and fused to preexisting muscle fibers as myoblasts regenerate the damaged skeletal muscle [128].

Skeletal Muscle Injury & the Immune Response

Background of Immune System Impact on Skeletal Muscle Injury

In 1957, the link between the immune system and skeletal muscle repair was exposed. By examining skeletal muscle post-injury, Godman [129] was the first to identify that leukocytes infiltrate the damaged tissue. Additionally, Godman found that leukocyte numbers stay elevated throughout muscle repair and regeneration thus suggesting that leukocytes may contribute to skeletal muscle regeneration [129].

To further investigate the possibility that damaged skeletal muscle may trigger an inflammatory response, Robertson, et al. in 1993 used Boyden chambers to examine the chemoattractant properties of crush-injury skeletal muscle on both polymorphonuclear leukocytes (PMLs) and macrophages. Additionally, Robertson, et al. also investigated the chemoattractant potential of macrophages and known macrophage cytokines on satellite cells using Boyden chambers. For this study PMLs and macrophages were collected from peritoneal washings post brewers thioglycollate broth injections. Satellite cells were obtained from the mouse C2C12 cell line and primary cultures from mouse hindlimb and back skeletal muscle.

Similar to the findings by Godman [129], Robertson, et al. [130] found that damaged myofibers release chemotactic factor(s) that attract both PMLs and macrophages. Furthermore, macrophages, but not PMLs, increased satellite cell migration and proliferation. When individual macrophage secreted products were examined, it was found that PDGFβ, FGF and TGF-β were mitogenic but PDGFα and LIF did not have the same mitogenic effect [130].

Since the Robertson et al. [130] study, more knowledge has been gained on the influence of the immune system in skeletal muscle repair. It is now known that following

skeletal muscle injury, an acute inflammatory response is triggered during the degeneration phase. The inflammatory response to muscle damage is dependent on both the degree of the muscle damage and the vascularization of the injured skeletal muscle at the time of injury [78]. However, in all cases, white blood cells including neutrophils, monocytes/macrophages, T cells and B cells migrate to the damaged muscle. This response augments the clearing of necrotic tissue and cellular debris during the while also stimulating the repair of myofibers, blood vessels, nerve fibers and the extracellular matrix. The immune response also attracts and activates other cell types critical for skeletal muscle repair such as fibroblasts and MPCs during the regeneration phase [15].

Prior research has also confirmed the importance of inflammatory cells with skeletal muscle repair. For instance, when non-steroidal anti-inflammatory drugs are administered to rats that inhibit macrophage and neutrophil function, there is impaired muscle regeneration that leads to weaker musculotendinosus units compared to untreated animals [40, 78].

Immune System Activation

As discussed, when muscle trauma occurs there is an extensive disruption of the muscle structural components during the degeneration phase. Normal sarcomeric organization is lost and myofibrils retract from the injury site. As a result, basement membrane and endomysium proteins are left surrounding the damaged area of the muscle fiber [78].

Additionally, the damaged skeletal muscle releases many factors that trigger an acute inflammatory response. These factors include adhesion molecules such as endothelial leukocyte adhesion molecule-1 (E-selectin), P-selectin, and intercellular adhesion molecule-1. These adhesion molecules are all chemoattractant muscle that

responsible for the influx of endothelial cells, neutrophils and monocytes to the site of injury. Furthermore, endothelial nitric oxide (NO) produced by endothelial nitric oxide synthase may also aid in infiltration of such cells due to the vasodilator properties of NO [15]. Stimulated endothelial cells also release IL-1α, IL-1β, IL-6 and IL-8 that are cytokine chemoattractants for granulocytes and macrophages. Within 2 hours of eccentric exercise induced injury the influx of white blood cells occurs [15, 131-135].

Neutrophils

The first type of white blood cells that infiltrate the damaged skeletal muscle tissue are neutrophils. Neutrophils arrive within the injury site immediately following injury and may remain within the skeletal muscle up to four days. Neutrophil concentration peaks between 1 to 4 hours after injury and then declines [26, 128, 136]. Neutrophils are a critical to the degeneration phase and serve to phagocytose necrotic myofibers and cellular debris. Neutrophils may also release pro-inflammatory cytokines such as IL-6, TGFβ and TNFα to help augment the inflammatory process [15, 17-21, 137]. Neutrophils also release IL-1 and IL-8 which are chemoattractant cytokines for macrophages [15].

Monocytes/Macrophages

Within 24 hours, the concentration of neutrophils within the damaged muscle decreases [26]. Following neutrophil infiltration, monocytes migrate to the damaged tissue. Monocyte migration occurs within 1-2 days following injury. Once within the skeletal muscle the monocytes mature and differentiate into tissue macrophages. Macrophages can remain within the skeletal muscle for 7-14 days and can be identified by their expression of specific cell markers such as ED1+ and ED2+ [15, 23-25, 30, 72, 138].

The ED1+ macrophages typically occur in greater concentration in the first four days of macrophage infiltration. The ED1+ macrophages release pro-inflammatory cytokines such as IL-1, IL-2, IFN-γ and TNF that stimulate the acute inflammatory process. ED1+ macrophages are also critical for removal of damaged myofibrillar material during the degeneration phase [15, 139-143]. These macrophages help remove cellular debris through phagocytosis. Macrophage phagocytosis is a remarkably specific and coordinated process in which cylinders of the basal laminas of the injured myofibers are left intact while the damaged tissue is removed via lysosomal enzymes. These preserved portions of the basal lamina later serve as scaffolds for satellite cells to form new myofibers [72, 144].

The appearance of ED2+ macrophages occurs around day four post-injury and aid in the regeneration phase. ED2+ macrophages are non-phagocytic but instead release growth factors and cytokines critical for regulating muscle precursor cell functions. ED2+ macrophages release anti-inflammatory cytokines including IL-4, IL-5, IL-6, IL-10 and IL-13. These macrophages help shift the injury site towards tissue repair with muscle growth and repair [15, 23-25, 72, 138]. IL-1 is a chemoattractant and activator for T and B cells [78, 145].

To further examine the role of macrophages with skeletal muscle regeneration, macrophage conditioned media has been studied. Conditioning media is an in vitro process in which cells of interest are activated then incubated in a basal media. Following incubation, the basal media is collected and is referred to as conditioned media since it contains the cytokines released from the activated cells. When MPCs were exposed to macrophage conditioned media, MPC proliferation and overall skeletal

muscle regeneration was enhanced following skeletal muscle injury [144, 145]. Additionally, it has been shown that macrophage depletion leads to increased fibrosis in regenerating skeletal muscle post-injury [146].

While there are resident fibroblasts within the muscle, additional fibroblasts are recruited. Cytokines such as IL-1, TNFα and TGFβ produced by neutrophils and macrophages stimulate fibroblasts to proliferate to repair damaged connective tissue and the extracellular matrix [147, 148]. Additionally, fibroblasts have also been shown to produce IL-6, IL-1α and IL-8 [149-151].

T Cells

Following the degeneration phase, the regeneration phase begins. However, depending on the injury, some degeneration processes may overlap with the regeneration phase. T cells, similar to ED2+ macrophages, increase during this time around 4-5 days post-injury [30]. A recent report by Cheng, et al. [30] examining post-injury immune cell infiltration found significant T cell infiltration from 5 days post-injury which continued past 10 days post-injury during skeletal muscle regeneration [30]. Thus, the time course of T cell infiltration overlaps with MPC proliferation and differentiation, as well as, the beginning of fibrosis [26, 27]. T cells can also produce anti-inflammatory cytokines such as and IL-4 and IL-10 that are linked to increasing satellite cell migration and the formation of myotubes [26, 27]. Considerable knowledge needs to be gained regarding the potential role of T cells in skeletal muscle regeneration.

Immune Lymphocytes

Developmental Lineage

All types of blood cells including red blood cells (RBCs, erythrocytes) and white blood cells (WBCs, leukocytes) are derived from pluripotent hematopoietic stem cells within the bone marrow. The pluripotent cells then divide to form either a common lymphoid progenitor cell or a common myeloid progenitor cell. Common lymphoid progenitor cells differentiate into the lymphoid lineage of leukocytes including natural killer (NK cells) and both T and B cells. Common myeloid progenitor cells differentiate into myeloid lineage cells that include erythrocytes but also the granulocyte leukocytes that include neutrophils, eosinophils, basophils, mast cells, monocytes and macrophages [152].

The lymphoid lineage of leukocytes contributes adaptive immunity. Adaptive immunity involves specific means of eliminating infections by recognition of the antigens. Antigens are any substance that can be recognized by the immune cells of the adaptive immune system. These include proteins, glycoproteins, polysaccharides, and even other chemical substances such as metals, drugs and organic chemicals of plants. When antigen recognition occurs, the adaptive immune system generates antibodies. B cells synthesize two types of antigen recognition molecules known as immunoglobulins. The immunoglobulins can be either secreted or bound within the cell membrane of the B cell surface. Membrane-bound immunoglobulins serve as B cell receptors for antigens. Antibodies bind directly to antigens in order to recruit other cells and molecules to destroy the antigen [152].

Lymphocytes circulate within the body through the blood and the lymph. They can also reside in high numbers within lymphoid tissues or lymphoid organs such as the

bone marrow, thymus, lymph nodes, the spleen and the mucosal lymphoid tissues of the gut. Both B and T cells originate in the bone marrow but only B cells mature within the bone marrow. Precursor T cells, comparatively, migrate to and mature within the thymus [152].

T and B cells are antigen-specific lymphocytes provide the immune system with an incredible ability to mount an immune response against a wide variety of pathogens. Both T cells and B cells undergo a maturation process upon exposure to antigens that allow them to specifically respond to a certain antigen. All lymphocytes start out as naïve cells that have not yet been activated by a specific antigen. Once a lymphocyte has been exposed to an antigen it further differentiates into a fully activated form known as an effector lymphocyte [152].

While both T and B cells are similar, they have different roles in the adaptive immune response. When a B cell binds an antigen to its cell membrane B cell receptor, the B cell proliferates and differentiates into effector cells known as plasma cells that produce antigen-specific antibodies. Thus, when an antigen activates a naïve B cell, the antigen becomes the target of the antibodies specifically created from the progeny of effector cells [152].

Throughout the course of an immune response some of the antigen activated B and T cells differentiate into memory cells that are responsible for long-lasting immunity towards specific diseases and pathogens. When a second exposure of an antigen occurs, memory cells can quickly differentiate into effector cells to increase the speed of the immune reaction [152].

T Cells

Similar to B cells, when naïve T cells are activated by a first encounter with an antigen the T cells proliferate and differentiate into one of many effector T cells. Cytotoxic T cells, helper T cells and regulatory T cells are the three types of effector T cells. Cytotoxic T cells are designed to kill cells infected with viruses or pathogens while helper T cells release cytokines to help antigen-stimulated B cells to activate, differentiate and produce antibodies. Helper T cells can also activate macrophages. Regulatory T cells are the third type of T cell effector that suppress lymphocyte activity and help control immune responses. When T cell development in the thymus ends all T cells are composed of two main classes characterized by either the CD8 or the CD4 cell-surface proteins. T cells with the CD8 protein develop into cytotoxic T cells while T cells with the CD4 protein generate helper and regulatory T cells. Furthermore, there are two main types of CD4 effector T cells termed Th1 and Th2 cells. Th1 cells recognize specific bacterial antigens displayed on macrophage surfaces, aid in macrophage activation and promote NK cell proliferation. Th2 cells also recognize specific antigens and stimulate the B cell activation and B cell antibody production [152].

The presence of certain cytokines during the initial stimulation can influence the development of either Th1 or Th2 T cells. When IL-12 and IFN-γ are present, then Th1 development is increased. If IL-2 is present, then Th2 development occurs. Each type of T cell also induces a different cytokine response. Th1 responses typically produce IL-2, IFN-γ, and TNF-α where Th2 responses generate IL-4, IL-5 and IL-10. For most situations there is typically a mix of both Th1 and Th2 activation but there are some situations that lead to a dominant production of either Th1 or Th2 cells [153].

T cells also have antigen-recognition molecules made solely as membrane bound proteins known as the T cell receptor that activates T cells. Even though T cell antigen-recognition molecules are similar to B cell immunoglobulins, the T cell receptor does not recognize and bind the antigen directly. Instead the T cell receptor recognizes short peptide fragments of protein antigens bound to the major histocompatibility complex (MHC) of antigen-presenting cells. There are two classes of MHC complexes: MHC class I and MHC class II. MHC class I molecules consist of two polypeptide chains which together form a peptide-binding cleft where the antigen will bind. The MHC class II molecules are also composed of two polypeptide chains that are folded together to form a wider peptide-binding cleft than the MHC class I molecules. The MHC class I molecules can only bind short peptides about 8-10 amino acids long whereas the MHC class II molecules can bind amino acids of any length [152].

CD8 T cells recognize the MHC I molecules while CD4 T cells recognize the MHC class II molecules. Effective T cell activation requires the binding of either the CD8 with the MHC class I molecule or the CD4 with the MHC class II molecule. Together, the CD4 with the MHC II and the CD8 with the MHC I are termed co-receptors. MHC class I molecules are mainly present on T cells, B cells, macrophages and neutrophils. MHC class II molecules are mainly present on B cells, macrophages, dendritic cells and epithelial cells of the thymus [152].

With in vitro T cell stimulation many mitogenic molecules and monoclonal antibodies can be used to induce proliferation. Common mitogenic molecules include phytohemagglutinin (PHA) and concanavalin A (Con A) which bind to cell membrane glycoproteins including the T cell receptor (TCR)/CD3 complex inducing activation.

Phorbol 12-myristate 13-acetate (PMA) also induces T cell activation via direct activation of protein kinase C [154].

For full lymphocyte activation to occur there must be two independent signals. The first signal is an antigen-specific signal that activates the unique antigen specific TCR. The second, co-stimulation signal and is a signal independent from the TCR [154]. When only the TCR is stimulated, T cells do not produce cytokines and cannot sustain proliferation. Furthermore, T cells activated solely by the TCR usually undergo apoptosis or become unresponsive to following stimulation. Co-stimulation can be thought of as a synergistic lymphocyte activation that does more than simply increase the TCR activation [154].

CD28 was the first co-stimulatory receptor to be identified. Since its identification, it is now known that CD28 is the primary co-stimulatory molecule for naïve T cells [154]. Thus, antibodies specific to the TCR/CD3 complex can also activate T cells but a co-stimulatory signal via CD28 is required to induce proliferation. Using anti-CD3/CD28 is more physiological than the use of mitogenic molecules since it mimics the binding of antigen presenting cells (APC) to T cells [154]. Once activated, T cells synthesize their principal growth factor IL-2 and the IL-2 receptor. The IL-2 receptor binds IL-2 which induces T cell proliferation [155]. Thus, in order to increase T cell activation, co-stimulation via the TCR/CD3 complex with CD28 plus exogenous IL-2 can enhance T cell proliferation [156, 157].

Immune Cell Cytokines

Pro-inflammatory Cytokines

Cytokines are regulatory proteins of low molecular weights that are produced and secreted by white blood cells and many other body cells in response to various stimuli. Cytokines are soluble glycoproteins that regulate cell-to-cell communications. Cytokine production can be induced by a variety of stimuli including infection, trauma and physical activity. Additionally, cytokines can be either inflammatory or anti-inflammatory in their physiological action [26, 158].

Pro-inflammatory cytokines include IL-1, IL-2, IL-6, TNFα and IFNs. Many immune and non-immune cells can produce IL-1. IL-1 is a pyrogenic cytokine in that it can induce fever, hypotension, lethargy and inflammation. IL-1 may also be associated with overtraining. IL-1 can inhibit both collagen and proteoglycan formation. It may also stimulate protein breakdown and inhibit protein synthesis [159]. Additionally, IL-1 amplifies T cell activation by stimulating the gene expression of IL-2 and IL-2 receptor. IL-1 also increases IL-6 production that stimulates B cell growth [153, 158].

IL-2 is another inflammatory interleukin. It is produced solely by T cells and serves in many inflammatory processes via its ability to activate both T cells and B cells. The IL-2 receptor (IL-2R) is composed of three subunits: IL-2Rα, IL-2Rβ and IL-2Rγ. Individually, IL-2Rα has a low affinity for IL-2. Joined together, IL-2Rα and IL-2Rβ have an intermediate affinity for IL-2. When all three subunits are joined they form the high affinity receptor [153, 158, 160].

IL-2 activates resting T cells and can act on T cells in both an autocrine and/or a paracrine manner. Naïve, T cells express the IL2Rα but only 1-6% co-express α and β

receptor subunits. Once activated with a mitogen or antigen, IL-2Rα and IL-2Rβ are strongly co-expressed. IL-2 also induces natural killer cell maturation into lymphokine activated killer (LAK) cells [153, 158, 160].

IL-6 is also an inflammatory cytokine that is an endogenous pyrogenic cytokine. It is produced by T and B lymphocytes, natural killer cells and monocytes. IL-6 is also produced by non-immune cells such as smooth muscle cells and skeletal muscle cells. IL-6 increases IL-1 and TNF production and is a potent mediator of the acute inflammation response. IL-6 also serves as a co-stimulator for T cell activation and B cell maturation. IL-6 is upregulated by IL-1, IL-2, IFNs and TNFα [19, 25, 26, 136, 158].

TNFα is also a pro-inflammatory cytokine that shares many physiological roles with IL-1 and IL-6. TNFα is mainly produced by mononuclear phagocytes but can also be synthesized by T lymphocytes, Kupffer cells, neural cells and endothelial cells. TNFα increases various cell surface adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin. These adhesion molecules all facilitate leukocyte infiltration at a site of injury. Additionally, TNF is a potent endogenous pyrogen [150, 158].

IFNγ is a pro-inflammatory interferon primarily produced by NK cells, Th1 and Th2 cells and cytotoxic cells. IFNγ is a macrophage activator and can induce non-specific cell-mediated defense mechanisms. IFNγ also increases other inflammatory cytokine synthesis such as TNFα and TNF receptors [30, 158].

Anti-inflammatory Cytokines

While inflammation protects an organism against infection and injury and is essential to injury repair, inflammation must subside in order to complete the full

restoration of normal tissue structure and function. The anti-inflammatory cytokines including IL-4, IL-10, IL-13, and IL-6 all serve to attenuate the inflammation via restriction of cytokine production and inflammatory cell activity. IL-4 is a cytokine produced by Th2 cells. IL-4 binds to a dimeric protein cell membrane receptor and facilitates the humoral immunity response and inhibits Th1 cells. IL-4 decreases antibody-dependent cell mediated cytotoxicity and other macrophage functions. IL-4 also attenuates the production of IL-1 and reduces NO production [158].

IL-6 is also considered an anti-inflammatory interleukin in addition to having pro-inflammatory properties. Unlike IL-1 and TNFα, IL-6 also does not activate neutrophils or macrophages or augment permeabilization of the vascular endothelium to aid in leukocyte extravasation. Instead, IL-6 depresses IL-1 and TNFα synthesis and attenuates additional IL-1 signaling by inducing IL-1 receptor synthesis and release. Together, IL-4 and IL-6 all decrease reactive oxygen species and nitrogenous intermediates that contribute to inflammation [19, 136, 158, 161].

IL-10 is another anti-inflammatory cytokine that plays a strong role in immuno-suppression that is mainly produced by the Th2 subset of leukocytes. IL-10 is secreted by macrophages, mast cells and B cells. Both IL-1 and TNF trigger a negative feedback response for inflammation activating IL-10 synthesis. Once produced, IL-10 inhibits pro-inflammatory mediator synthesis including IL-1, TNFα, and IL-12. IL-10 also lowers macrophage NO production and inhibits IFNγ production by T cells and NK cells. Overall, IL-10 decreases cell-mediated inflammation [158].

Cytokines may also stimulate satellite cell chemotaxis. PDGF-AB, PDGF-BB, FGF-2 and TGFβ have all been found to increase in vitro muscle precursor cell migration

[162]. Similarly, IL-4 has also been shown to increase myoblast migration [163]. Following chemotaxis, satellite cell proliferation and differentiation occurs. Currently, cytokine growth factors such as the acidic and basic forms of FGF, TGF-β, IGF and PDGF may significantly increase satellite cell proliferation [36, 49, 164, 165]. Additionally, FGF, PDGF and IL-1 all inhibit differentiation in comparison to IGF that enhances differentiation [36, 164, 166] (Appendix 3: Table 1).

Overview

Since 1961 considerable interest and research efforts have focused on the cell physiology of myogenic satellite cells. Despite such progress, there are still many fundamental questions that need to be explored [48]. Currently, both neutrophils and macrophages have been found to play a vital role in skeletal muscle repair, but the potential influence of lymphocytes, specifically, T cells, on MPCs has yet to be determined. Since T cells infiltrate damaged skeletal muscle and are a main producer of many cytokines, the role of T cells on MPC function must be examined.

Clinical Relevance of Research

Duchene Muscular Dystrophy

With dystrophinopathies, muscle damage is progressive. In Duchene muscular dystrophy (DMD) there is a lack of a functional dystrophin gene that encodes a cytoskeletal protein associated with the membrane. DMD muscle fibers become mechanically weaker and develop lesions during high intensity contractions. Unfortunately, in DMD, the replication capacity of MPCs is significantly reduced. Since DMD is characterized by cycles of skeletal muscle degeneration and regeneration, the reserve population of satellite cells is depleted and DMD skeletal muscle eventually

cannot regenerate [167, 168]. Eventually, DMD leads to a progressive loss of skeletal muscle.

When muscular damage occurs with DMD an immune response is triggered. Recent evidence has shown that T cells chronically infiltrate the damaged DMD muscle due to repeated cycles of degeneration and regeneration [27]. The infiltrating T cells have been implicated in the pathology of muscular dystrophy [27]. Recently, the use of immunodeficient/dystrophic mouse models (crossing a nude or SCID mouse with muscular dystrophic mice), demonstrated that T cell depletion resulted in both reduced TGF-β1 and skeletal muscle fibrosis in dystrophic skeletal muscle [33, 38]. These dystrophic/immunodeficient mice demonstrated improved skeletal muscle regeneration and decreased fibrosis [33, 38]. One possible interpretation of this study is that the chronic infiltration of T cells in the lesions of dystrophic skeletal muscle promotes the fibrotic aspects of muscular dystrophy. However, the role of T cells in skeletal muscle repair of non-dystrophic skeletal muscle is not known. A key difference is the relatively short window of accumulation of T cells and macrophages compared to chronic infiltration associated with the continuous degeneration and regeneration of muscle both in Duchene Muscular Dystrophy and in the mdx mouse. In non-dystrophic animals lacking T cells (nude mice), muscle growth is impaired [169].

Post-Space Flight

From spaceflights as early as the Apollo and Skylab, it has been known that astronaut immunity is impaired both during and following spaceflight [170]. Research from Spacelab 1 during the 6th mission of the Columbia space shuttle demonstrated that T and B cells proliferation is hindered from exposure to reduced gravity [171]. Additionally, decreased lymphocyte function in astronauts is likely the result of a

combination of stress and weightlessness [172]. More recent evidence suggests that the net effect of spaceflight (stress and microgravity) results in reduced T cell activation [173-176].

As mentioned, one specific cytokine of interest that plays a critical role in T cell activation is IL-2. T cell activation is marked by the production of IL-2 and the expression of the IL-2R. IL-2 is released by activated T cells and stimulates the production of T helper cells, natural killer T cells, and B cells. Interestingly, T cells obtained from mice that traveled in the STS-118 shuttle had decreased percentages of CD3+ T cells. When the same T cells were activated with an anti-CD3+ antibody they produced lower amounts of IL-2 compared to control mice [177]. Furthermore, T cells isolated from rats from the Space Shuttle Mission STS-57 had decreased IL-2 production when they were exposed to the T cell receptor-independent mitogen, 12-O-tetradecanoylphorbol-13-acetate (TPA) plus ionomycin (ION) [174]. In addition to the decreased production of IL-2, expression of the IL-2 receptor is has also been shown to be decreased following spaceflight in leukocytes from rhesus monkeys. Currently, the implications of decreased synthesis of IL-2 and IL-2R in T cell function is a highlighted area of research focused on impaired immunity both during and following spaceflight.

In addition to the reduced immune cell and T cell impairments that occur with spaceflight it is well documented that astronauts experience severe reductions in skeletal muscle mass during exposure to microgravity. Furthermore, upon return from spaceflight, skeletal muscle regeneration of astronauts is impaired [178-180]. Since evidence is increasing that immune cells, specifically T cells, are key regulatory cells in

skeletal muscle repair and growth, reduced T cell function may be a critical link in regaining skeletal muscle mass post-spaceflight.

MPCs & Aging

Aging is associated with a marked decreased skeletal muscle mass and strength with increased skeletal muscle fibrosis and adipose deposits within the muscle. Eventually, these impairments can lead to a condition known as sarcopenia. One contributing factor to sarcopenia is the diminished capacity for aged skeletal muscle to regenerate [43], hypertrophy [51] and regrow after a bout of atrophy [20]. As a result, sarcopenia is associated with decreased quality of life and increased mortality in our aging population [22].

As previously mentioned, the resident stem cells responsible for skeletal muscle repair are termed MPCs. Recent evidence suggests that an age-associated loss of satellite cell functionality is the primary factor responsible for the loss of regenerative potential and increased atrophy and fibrosis of aged skeletal muscle (8). Current studies have provided evidence of impaired MPC activation [9, 11] proliferative capacity [10, 11, 48, 181] and differentiation with aging [10, 182]. Overall, satellite cell/MPC function is dictated by both the environment [183, 184] and intrinsic MPC dysfunction [8-12].

To determine whether age-related skeletal muscle regeneration impairment is dependent upon the age of the skeletal muscle or the surrounding millieu, Bruce & Faulkner [183] conducted whole extensor digitorum longus (EDL) muscle transplantations with young and old rats. After 60 days of recovery, the transplanted EDL muscle were removed and mass and force production were assessed. The cross-age transplantations showed that EDLs isolated from old rats regenerated at the same capacity within young rats as young EDLs grafted into young rats. Furthermore, young EDL grafts

in old rats did not regenerate better than old EDL grafts transplanted into old rats. For both cross-age transplants, the age of the grafted muscle did not dictate the regeneration capacity while the age of the recipient animal did [183].

In addition to changes in external stimuli, intrinsic MPC functioning in response to environmental factors have been discovered. For example, Schultz and Lipton [181] isolated satellite cells from hindlimb muscles of rats spanning between 6 days and 30 months of age. All satellite cells were cultured under the same conditions and were carefully monitored every 24 hours for 4-7 days. From this study it was found that proliferation rates significantly decreased with increasing age. Since the cells were cultured under the same in vitro conditions, this study supports the concept that satellite cells have intrinsic functioning impairments with age [181].

Similarly, Jump et al. [7] tested the responsiveness of MPCs isolated from young (3-month old) and old (32-month-old) rats to FGF2. Despite identical treatment conditions with FGF2, proliferation of the old MPCs was significantly less than the young MPCs. Together these studies indicate that MPC intrinsic function is impaired with aging despite external conditions [7, 181].

T Cells & Aging

Since 1967, age associated changes in the immune system have been observed [160, 185]. The most noted change that occurs with age in the immune system is the hindered capability of T cells to activate and proliferate in response to stimuli [186-188]. Additionally, there is a shift towards more T cells with activated/memory phenotypes with less naïve T cells [189, 190]. However, following functional maturation of the immune system, thymus involution occurs. This leads to a significant reduction of thymic tissue a reduction in naïve T cell production [160].

While most pro-inflammatory cytokine circulatory levels remain unchanged or increase with increasing age, the responsiveness to the T cell activating cytokine, IL-2, diminishes with age. Shinkai et al. [191] demonstrated that in vitro stimulation of blood collected from healthy elderly individuals had normal IL-1β, IL-4 and IFNγ production but impaired IL-2 response compared to young individuals. In vitro work has illustrated that CD4 T cell mediated responses decrease with increasing age [191]. Furthermore, aged CD4 T cells produce less IL-2 and express less of the IL-2 receptor [192-195]. As a result, aged T cells have reduced proliferation and impaired differentiation into Th1 or Th2 effector cells [190]. The reduction in T cell activation with age has been found in humans, mice and rats and with external stimuli including PHA, ConA, anti-CD3 and IL-2 [160, 196, 197].

Impairments in molecular cell signaling have also been found in aged T cells. Such events include phospholipid hydrolysis, protein kinase C activation and translocation and increases in the concentration of cytosolic calcium [160]. Together these age-associated cell signaling changes lead to impaired CD4 T cell activation [198].

Furthermore, studies have also compared the cellular immune function of sedentary aged individuals to aerobically conditioned elderly individuals. From these studies both NK cell activity and lymphocyte proliferation in response to PHA have been higher in individuals who are aerobically trained [198, 199]. Additionally, T cells obtained from active elderly individuals have been shown to have superior T cell proliferation, and IL-2, IFNγ, ad IL-4 synthesis compared to sedentary aged individuals [199]. Animal studies have also supported these findings. Nasrullah and Mazzeo found

that 15 weeks of aerobic training in elderly rats increased the T cell proliferative response and IL-2 production [158, 200].

In order to provide new treatments for DMD, skeletal muscle regeneration post spaceflight and sarcopenia, studies examining the regulation of MPC function must be conducted. A better understanding of the mechanisms that regulate MPC function and lead to skeletal muscle repair is required. Furthermore, since the impact of immune cells on MPC function is beginning to be determined and the impact of T cells on MPCs has yet to be studied, research should focus on the potential influence of T cells on MPC function.

Chapter V

Study Design

Animals

All experimental animal procedures were approved by the Colorado State University Institutional Animal care and Use Committee. For all experiments, 3-month-old Fisher and 32-month-old 334 X Brown Norway F1 hybrid male rats were used obtained from the National Institute on Aging. Animals were housed at 21°C, maintained on a 12 to 12 hour light-dark cycle, and allowed free access to food and water. The animals were killed by intraperitoneal injection of ketamine (80 mg/kg), xylazine (10 mg/kg) and acepromazine (4 mg/kg).

Experimental Design

Cell culture experiments utilizing isolated MPCs and T cells were performed to gain insight into the signaling from T cells to MPCs that may be critical to MPC function. It was hypothesized that conditioned media containing cytokines released from activated T cells will increase proliferation, differentiation and migration of MPCs.

Limitations and Basic Assumptions

Although the conducted research provides beneficial insight into the crosstalk between MPCs and T cells, unfortunately the research was conducted on rat cells instead of human cells. Thus, although rat and humans are very similar genetically in that there are many conserved sequences between the two species, the research should only be used as a beginning foundation for understanding cell physiology within humans.

Additionally, there are some limitations to working with cell culture experiments. In order to stimulate the T cells, IL-2, anti-CD28, and anti-CD3 were added to the cultures to provide full stimulation. Although IL-2 and both antibodies were successful in increasing T cell proliferation, it is currently unclear as to what cytokines or released biomolecules trigger T cell activation at the site of muscle injury. Also, it should be mentioned that the following studies only address T cell and MPC interactions. Thus, it is possible that interactions between immune cells and MPCs are only one part of the puzzle involving T cell and MPCs function. Additionally, since the experiments were conducted in vitro, many environmental elements of the in vivo niche are missing such as connective tissue and skeletal muscle fibers.

Methods.

MPC Isolation

Muscle precursor cells were isolated following the method by Allen et al. [65] modified by Lees et al. [182]. Briefly, the gastrocnemius and plantaris of both hind limbs were dissected from each animal. The muscles were then minced and cells were isolated by pronase digestion. Following pronase digestion, the cells were pre-plated for 2 hours and then for 24 hours on tissue culture-treated 150 mm in diameter plates. Following the 24 hour pre-plating step, non-adherent cells were seeded onto 150mm diameter plates coated with Matrigel (0.2 mg/ml; BD Biosciences, San Jose, CA) for 1 hour at 37°C. The cells were then cultured for 3-4 days in growth medium (GM) containing 20% fetal bovine serum (FBS; Lot # KTH31753 Hyclone, Thermo Fisher Scientific, Waltham, Massachusetts), 100 μL/ml penicillin, 100 μg/ml streptomycin, and 40 μg/ml gentamycin in Ham's F-10 medium (HyClone, Thermo Fisher Scientific, Waltham, Massachusetts) in

a HERACell 150i humidified incubator (Thermo Scientific, Waltham, Massachusetts) set to 6% O_2, 5% CO_2, and 89% N_2 at 37°C (oxygen was increased to 6% versus 5% in previous studies [37] to compensate for the influence of altitude) until 80% confluence of the cells was reached. The cells were collected and stored at approximately 1,000,000 cells per vial and frozen in the vapor state of liquid nitrogen (Figure 1).

T Cell Isolation

Using sterile dissection technique, the spleen of each animal was removed and placed in a 15 ml conical containing 10 ml of Dulbecco's Modified Eagle Media (DMEM; ATCC, Manassas, Virginia). The dissected spleen was then transferred to a 100 mm in diameter plate and crushed with the plunger of a 10 ml syringe. The media was then collected in a 25 ml serological pipet and strained through a cell strainer (100 μm Nylon Mesh Sterile Cell Strainer, Thermo Fisher Scientific, Waltham, Massachusetts) to isolate splenocytes from spleen tissue fragments. The plate was then washed two times with 10 ml of DMEM that was collected, strained, and added to the total splenocyte/DMEM volume. Collected splenocytes were then split equally between two 15 ml conical vials and centrifuged at 300 x g for 10 minutes. Supernatant was discarded and each cell pellet was re-suspended in 2 ml of 2% FBS in Dulbecco's phophate buffered saline (DPBS; Cell Grow, Mediatech, Manassas, Virginia) (Figure 2).

T Cell Enrichment using Dynabead Separation

To isolate CD3 T cells, Dynabead indirect cell isolation technique was used. For each vial of collected splenocytes, 40 μl of mouse monoclonal antibody to rat CD3 (CALTAG Laboratories, Invitrogen, Carlsbad, California) was added and incubated for 20 minutes at 4°C on an orbital shaker. Following incubation with anti-CD3 cells were centrifuged at 300 x g for 10 minutes and re-suspended in 4 ml of DPBS with 0.1%

bovine serum albumin (BSA) and 2 mM of ethylenediaminetetraacetic acid (EDTA). Each vial was split again into two new 15 ml conical vial and 50 μL of pre-washed rat anti-mouse IgM Dynabeads (Dynal, Invitrogen, Oslo, Norway) was added to each vial. All four vials were incubated for 45 minutes at 5°C. After incubation, CD3 cells were isolated using the Dynabead magnet and then re-suspended in 10 ml OpTmizer T-cell Expansion serum free media (Gibco, Invitrogen, Carlsbad, California). OpTmizer media was prepared as specified by manufacturer's directions and was composed of OpTmizer T-Cell Expansion Basal Medium, OpTmizer T-Cell Expansion Supplement, 2 mM L-glutamine, 100 uL/ml penicillin and 100 μg/ml streptomycin. To stimulate the T cells 100 IU/ml of IL-2 (PreproTech, Rocky Hill, New Jersey) and 5 μg/ml of anti-CD28 (BD Biosciences, Pharmingen, San Diego, California) was used. T cells were plated onto 100 mm plates at a density of 5,000,000 cells per plate and cultured for 48 hours in the humidified HERAcell 150i incubator set to 6% O_2, 5% CO_2, and 89% N_2 at 37°C.

T Cell Condition Media.

Following the 48 hour incubation in stimulating media, cell plates were removed from the incubator and gently shook side to side for five times to remove the semi-adhesive T cells. The cells were then collected in a 50 ml conical vial via 25 ml serological pipet. The plate was then washed with 10 ml of 2% FBS in DMEM and collected in the same 50 ml conical. The cells were centrifuged at 200 x g for 10 minutes, supernatant was discarded and 10 ml of DPBS was added carefully not to disturb the cell pellet. Cells were centrifuged again at 200 x g for 5 minutes, supernatant was removed and cells were re-suspended in 10 ml 2% FBS in DMEM. After incubation, conditioned media was collected via 10 ml serological into a 15ml conical vial,

centrifuged at 200 x g for 10 minutes and poured off into a new 15 ml conical vial to remove the cells. Conditioned media was frozen at 20° C.

T Cell Proliferation Analysis Via BrdU Incorporation.

To examine the in vitro activation of T cells via anti-CD3, IL-2 and anti-CD28, the incorporation of 5-bromo-2'-deoxyuridine-5'-monophosphate (BrdU), a synthetic analogue of thymidine, was analyzed. Following the 48-activation incubation, the cells were pulsed labeled with BrdU by for 45 minutes in the cell culture incubator. Post-pulsing, cells were washed two times with 10 ml of DPBS then incubated for 5-10 minutes with 3ml of TrypLE Express (Gibco Invitrogen, Carlsbad, California) until 95% of all cells visibly under magnification released from the plate. Cells were collected from each plate and transferred into in a 15ml conical vial. Each plate was washed with 6ml pre-warmed GM and was collected as well. Cells were centrifuged at 200 x g for 5 minutes, supernatant was discarded and the cell pellet was re-suspended in 200 μL of DPBS. Single-cell suspension was achieved by a quick vortex followed by trituration. Cells were fixed via the addition of 4 ml ice-cold 70% ethanol and stored at 4°C.

Thawing MPCs.

For experiments, all MPCs were thawed via submersion in a Bath Armor bead (Lab Armor, San Antonio, Texas) bath set to 37°C. Thawing was carefully monitored and the cell vial was removed from the Bath Armor bath when only a small trace of ice remained. Once the sample completely thawed at room temperature, the MPCs were slowly diluted to 100,000 cells per ml via drop-wise addition of Ham's F10. MPCs were diluted again to a final concentration of 50,000 cells per ml in 10% FBS Ham's F10 by addition of 10 ml of 20% FBS Ham's F10.

MPC Proliferation.

To examine the influence of activated T cells on MPC proliferation, MPC cultures were cultured with conditioned media. For all proliferation experiments, cells were seeded at 125,000 cells per plate via addition of 2.5 ml of thawed cell stock and an additional 7.5 ml of 10% FBS Ham's F10 to each 100 mm in diameter plate pre-coated with 0.1 mg/ml of Matrigel. Plates were cultured for 24 hours in the HERAcell incubator.

Following the 24-hour incubation, media was aspirated off and 4.5ml of 20% FBS Ham's F10 and 4.5 ml of either conditioned media or basal media (2% FBS in DMEM) was added to each plate. For each set of proliferation experiments, the MPCs from an animal were used with conditioned media collected from the stimulated T cells of the same animal. Cells were then incubated for 24 hours.

Satellite Cells BrdU Pulsing & Fixing.

To analyze satellite cell proliferation, the incorporation of BrdU was analyzed. After 23 hours of exposure to either conditioned media or basal media, the cells were pulsed labeled with BrdU by for 45 minutes in the cell culture incubator. Post-pulsing, cells were washed two times with 10 ml of DPBS then incubated for 5-10 minutes with 3 ml of TrypLE Express (Gibco Invitrogen, Carlsbad, California) until >95% of all cells were visibly released from the plate under magnification. TrypLE was collected from each plate, placed in a 15 ml conical vial. Each plate was washed with 6ml pre-warmed GM and was collected as well. Cells were centrifuged at 200 x g for 5 minutes, supernatant was discarded and the cell pellet was re-suspended in 200 μL of DPBS. Single-cell suspension was achieved by a quick vortex followed by trituration. Cells were fixed via the addition of 4 ml ice-cold 70% ethanol and stored at 4°C.

Acid Denaturation & Flow Cytometry.

Post-fixing, cells were prepped for flow cytometry first denaturing the cell DNA using 4 M hydrochloric acid (HCl). First, fixed cells were centrifuged at 200 x g for 15 minutes. Supernatant was removed and cell pellet was gently re-suspended in 667 µL of DI H2O. 333 µL of 6 M HCl was then added, solution was vortexed and allowed to incubate for 30 minutes at room temperature. 2 ml of borate buffer was added and cells were centrifuged at 200 x g for 10 minutes. Supernatant was removed and cells were washed with 1ml of DPBS. Cells were spun down again at 200 x g for 10 minutes then 50-100 µL of anti-BrdU fluorescein in DPBS with 0.1% BSA was added to the cells and incubated for 30 min. 20,000 cells were analyzed using a Epics XL-MCL Coulter flow cytometer (Beckman Coulter, Brea, CA) and FCS Express Software (De Novo Software, Los Angeles, CA) [8]. Gating parameters were set by side and forward scatter to eliminate dead and aggregated cells.

MPC Migration

Following the first passage, MPC migration was measured using the FluoroBlok cell migration plates (8 µm pore size; BD Biosciences, San Jose, CA) with inserts coated with Matrigel. 75,000 MPCs were loaded into the upper chamber of the migration plate in BM containing calcein-AM (5 µg/ml). The lower chamber contained either basal media, or conditioned media obtained from T cells. The migration plate was incubated in the HERACell 150i humidified incubator set to 21% O_2, 5% CO_2, and 89% N_2 at 37°C (oxygen was increased to 21% versus 6% due to increased media depth within the dual well chambers). To detect the rate of migration, the plates were read with bottom reading fluorescence (Ex485/Em530) every 30 minutes up to 120 minutes (Soft Max Pro Software; Spectra Max M5, Molecular Devices, Silicone Valley, CA).

Appendices

Appendix 1: Manuscript Figures & Graphs

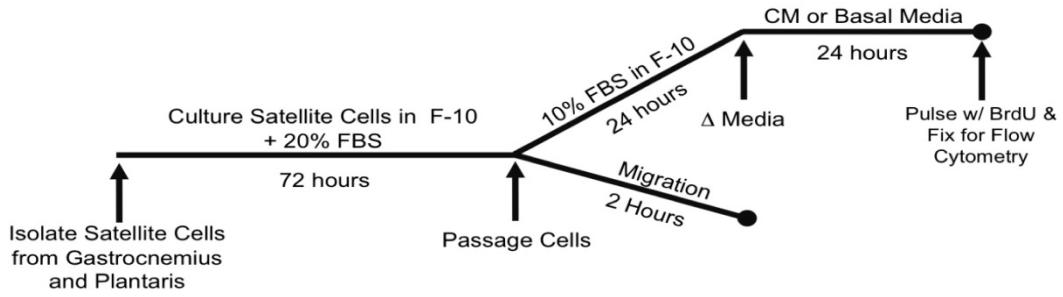


Figure 1:

Experimental timeline for satellite cell isolation, migration and proliferation assays.

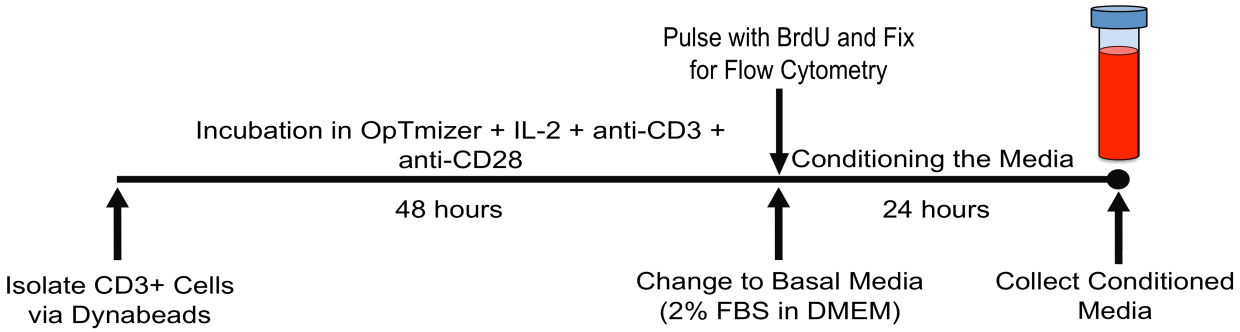
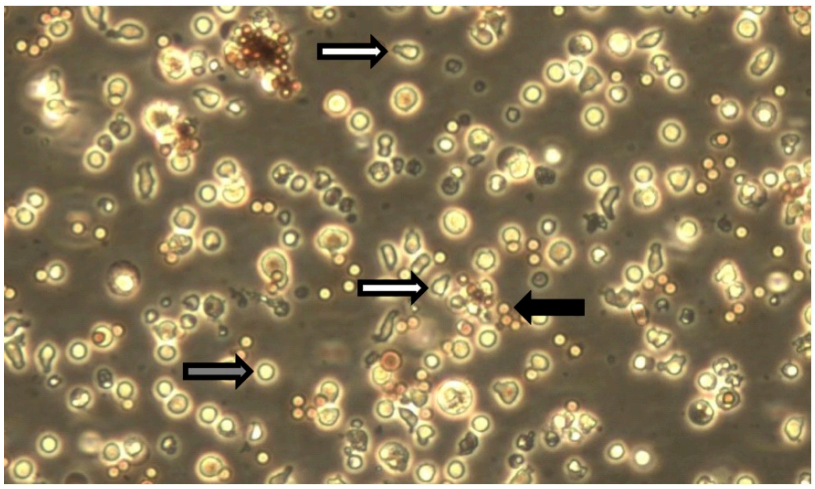


Figure 2: Experimental design for conditioning media from activated T cells.

A.



B.

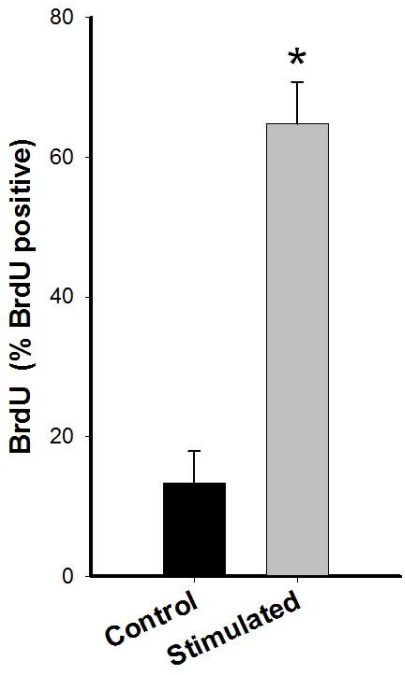
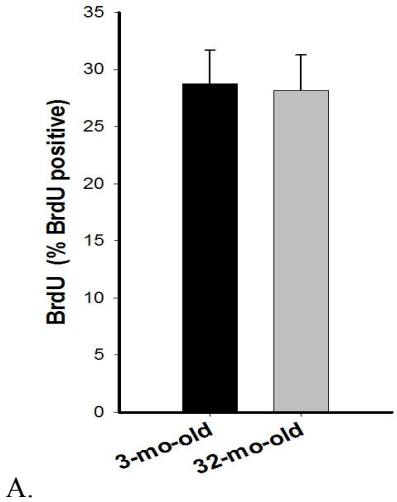
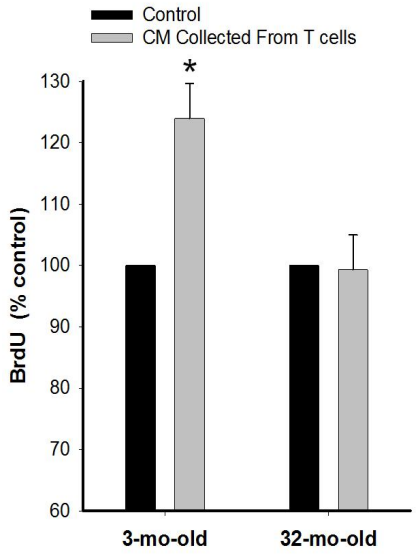


Figure 3: In vitro stimulation of isolated CD3+ T cells. A) CD3+ T cells (T cells with "hand-mirror" morphology labeled by the white arrows and spherical T cells labeled by the grey arrow) from 3-mo-old Fisher 344 X Brown Norway rats (n=4) were isolated via Dynabeads (black arrow) and cultured in OpTmizer media. B) T cell stimulation was performed for 48 hours with 100 IU/mL IL-2 and 5ug/mL of anti-CD28. Pulsing with 5-bromo-2'-deoxyuridine-5'-monophosphate (BrdU), a thymidine analog, was performed in the final hour of incubation to assess proliferation. Treatment with Il-2 and anti-CD28 increased T cell proliferation by 3-fold compared to T cell proliferation of control isolated CD3+ T cells that did not receive IL-2 and anti-CD28 treatment (67.77 $\pm$6.02 and 13.38 $\pm$4.55, respectively) *Denotes significance of a p value > 0.05.



A.



B.

Figure 4: Influence of T cell conditioned media on satellite cell proliferation in satellite cells isolated from 3-mo-old and 32-mo-old Fisher 344 X Brown Norway rats (n=4). Conditioned media (CM) collected from activated CD3+ T cells 24 hours following the 48-hour stimulation with Il-2 and anti-CD28. Satellite cell proliferation was assessed by flow cytometer assessment of the incorporation of 5-bromo-2'-deoxyuridine-5'-monophosphate (BrdU), a thymidine analog. A) 3-mo-old and 32-mo-old satellite cell proliferation in basal media. Proliferation was not significantly different in basal media between 3-mo-old and 32-mo-old satellite cells. B) CM significantly increased proliferation of the satellite cells isolated from the 3-mo-old rats compared to control (35.52$\pm$3.70 and 28.74 $\pm$2.98, respectively). CM did not significantly increase proliferation of the satellite cells isolated from the 32-mo-old rats (27.49 $\pm$2.34 and 28.19 $\pm$3.09, respectively). *Denotes a p value greater than 0.05.

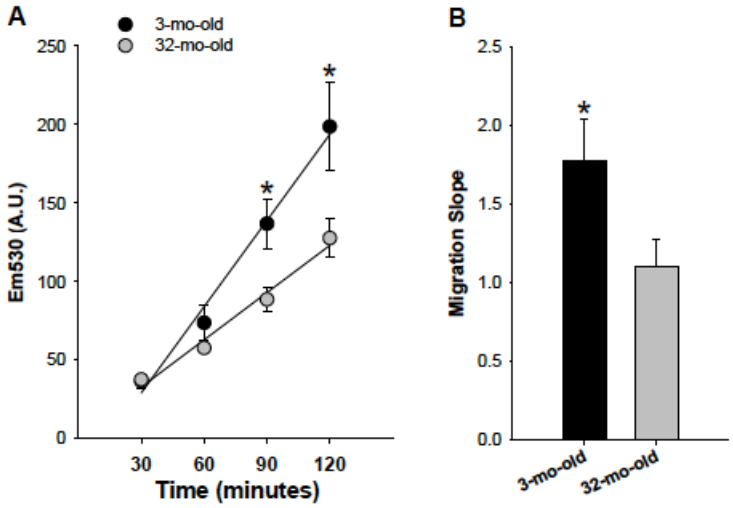


Figure 5: Satellite cell migration in basal media. A) Migration of satellite cells isolated from 3-mo-old and 32-mo-old Fisher 344 X Brown Norway rats (n=4 and n=4, respectively) was assessed in basal media (2% FBS in ATCC DMEM). Both satellite cells isolated from the 3-mo-old rats and 32-mo-old rats migrated in basal media. However, satellite cells from the 32-mo-old rats displayed impared migration compared to the migration of the satellite cells from the 3-mo-old rats. B) Rate of migration was calculated for both sets of satellite cells. The satellite cells from 3-mo-old rats migrated at a significantly faster rate (1.78 $\pm$ 0.26) compared to the satellite cells from the 32-mo-old rats (1.10 $\pm$ 0.17). *Denotes a p value > 0.05.

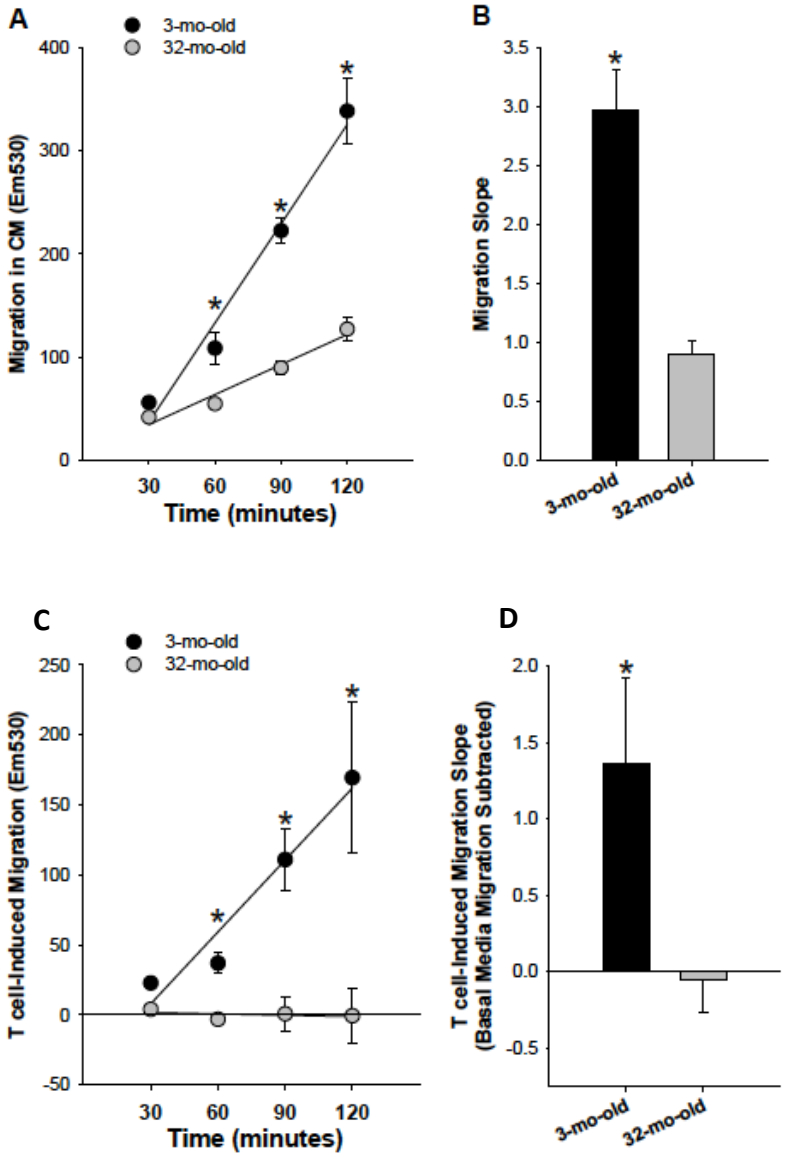


Figure 6: Migration of satellite cells to conditioned media. A) To test the chemoattractant properties of conditioned media (CM) from activated CD3+ T cells, migration was recorded at 30 minute time points for 120 minutes for satellite cells isolated from 3-mo-old and 32-mo-old Fisher 344 X Brown Norway rats (n=4 and n=4, respectively). D) Migration rate was calculated from the migration in CM data. The migration rate of satellite cells from 3-mo-old rats was significantly greater than the satellite cells from the 32-mo-ol rats (2.97 $\pm$0.35 and 1.05 $\pm$ 0.18, respectively). (C) Since migration of satellite cells was observed with basal media, the migration of satellite cells with migration media was subtracted from the migration data with CM. Migration of the satellite cells from the 3-mo-old rats migrated at a greater level than the satellite cells from the 32-mo-old rats. Migration values were significantly different at 60, 90 and 120 minutes. D) The rate of migration was calculated from the overall migration data. The migration rate of satellite cells from 3-mo-old rats was significantly greater than the satellite cells from 32-mo-old rats (1.36 $\pm$ 0.56 and 0.42 $\pm$ -0.05, respectively). *Denotes significance of p>0.05.

Appendix 2: Literature Review Figures & Graphs

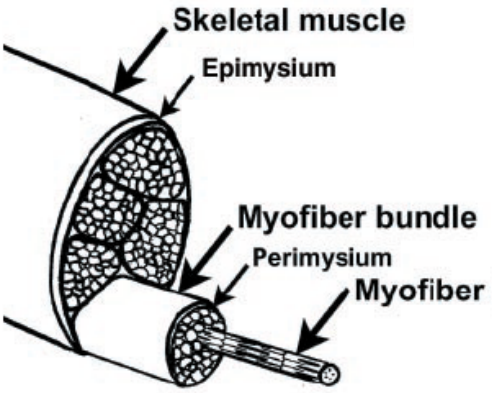


Figure 7. Organization of a mammalian adult skeletal muscle. Myofibers are individually surrounded by the endomysium and then grouped together by the perimysium to form a myofibers bundle, also known as a fascicle. Myofiber bundles are grouped together by the epimysium to compose the skeletal muscle [71].

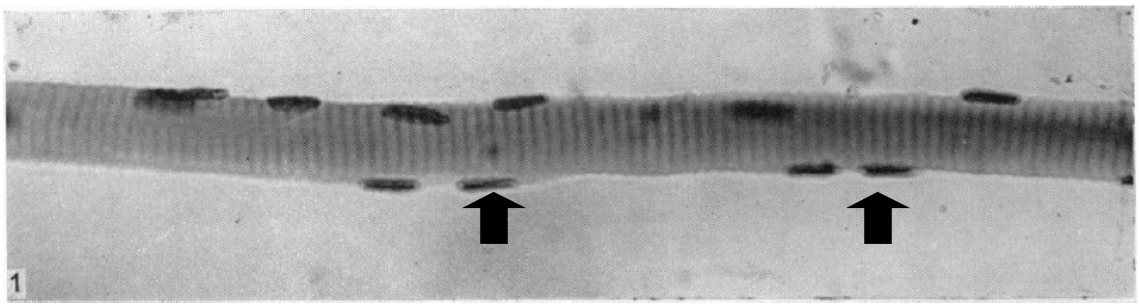


Figure 8. Isolated skeletal muscle fiber from a fruit-bat wing with labeled nuclei. Black arrows are pointing to the nuclei of satellite cells [47].

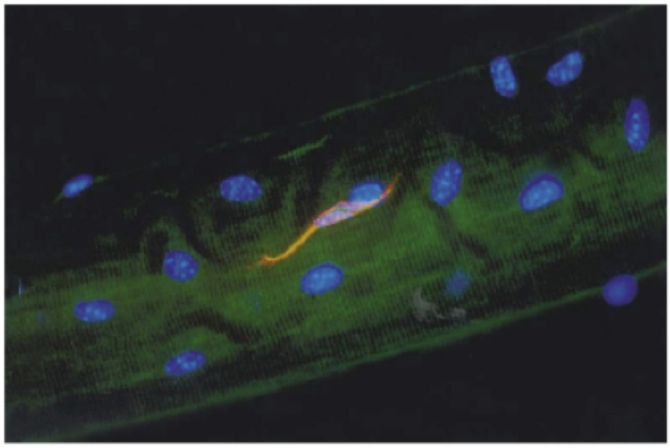


Figure 9. Muscle precursor cell (MPC) on an isolated skeletal muscle fiber. The muscle is stained with M-cadherin (red) and desmin (green) and counterstained with DAPI (blue) for nuclei staining [48].

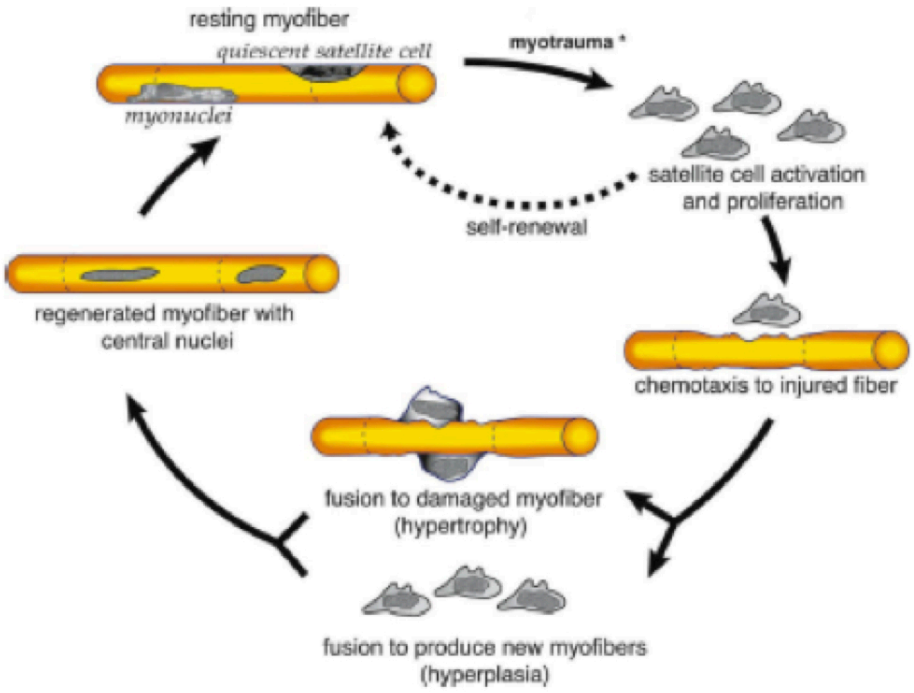


Figure 10. The repair of skeletal muscle damage by muscle precursor cells (MPCs). When skeletal muscle injury occurs, quiescent MPCs are activated and proliferate. A subset of the newly proliferated satellite cells will become quiescent to self-renew the inactivated satellite cell pool while some of the new progeny will migrate to the injured fiber(s). Depending on the severity of the injury the MPCs will either fuse with the preexisting injured myofiber(s) to repair the damage or fuse to produce new myofibers [48].

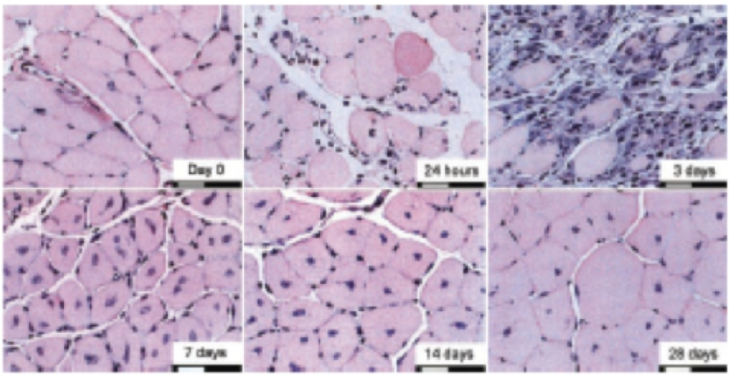


Figure 11. Cross-sections of mouse skeletal muscle post-ischemic injury. Despite the significant amount of intramuscular inflammation and the atrophy of skeletal muscle fibers observed between 24 hours and 14 days, skeletal muscle fiber size is regained by 28 days. By 28 days, histological findings show that the skeletal muscle has fully regenerated from the FAL injury [201].

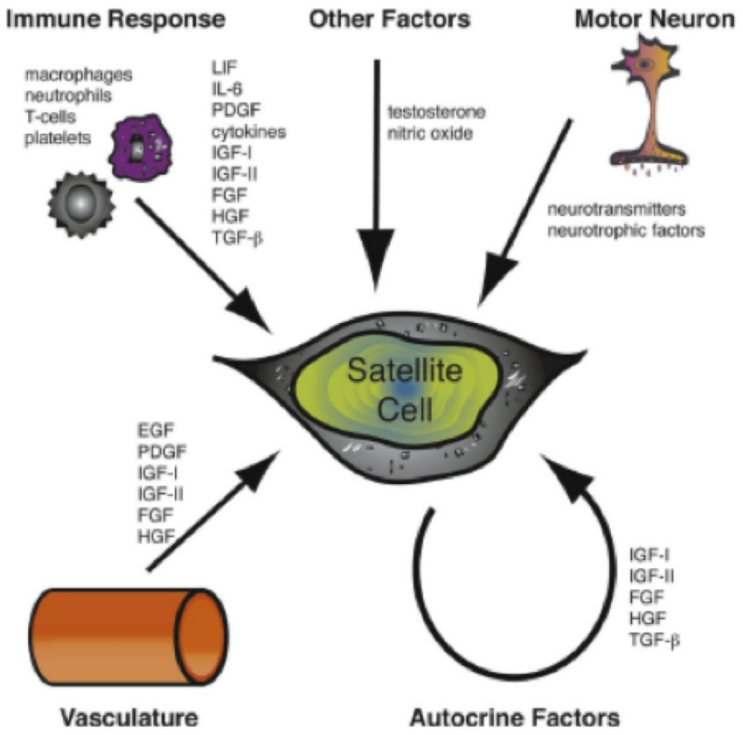


Figure 12. External regulation of muscle precursor cell (MPC) activity. Mitogens and cytokines that regulate MPC activation, proliferation, migration and differentiation are released from immune cells, motor neurons, other MPCs and the surrounding vasculature [48].

Appendix 3: Literature Review Tables

Growth Factor	Chemotactic Ability	Proliferation	Differentiation
FGF		+	
HGF	+	+	_
IGF-I		+	+
IGF-II		+	
TGF-β	+	+/-	+/-
Macrophages		+	+
LIF	+	+	
IL-6		+	
Basic PDGF	+	+	
EGF		+	
IL-1			-

Table 1: The mitogenic & chemoattractant abilities of growth factors and cytokines [48, 159].

Appendix 4: Data Tables

Table 1: T Cell Proliferation		
	Condition	
T Cell	**Control**	**IL-2 & anti-CD28**
40036	16.84	71.64
40037	28.15	73.32
3-902	0.83	40.94
3-903	8.25	68.51
3-904	12.84	69.44
Mean	13.382	64.77
SEM	4.55	6.02
% increase		384.01
Fold-increase		4.84

Table 2: MPC Proliferation				
Young		**Condition**		
MPC		**Control**	**CM**	**% increase**
Y813		22.61	30.54	35.07
Y811		32.39	42.85	32.29
3-901		24.85	27.92	12.35
3-902		35.1	40.78	16.18
	Mean	28.74	35.52	23.98
	SEM	2.98	3.70	5.69
Old				
MPC		**Control**	**CM**	**% increase**
O812		22.81	24.41	7.01
O812		30.49	31.29	2.62
O814*		35.87	31.67	-11.71
O807		23.58	22.58	-4.24
	Mean	28.19	27.49	-1.58
	SEM	3.09	2.34	4.09

Table 3: Migration - Young				
Animal	**Time (min)**	**C-2% FBS DMEM**	**Conc. CM**	**-Control**
3-905	30	42.66	63.59	20.93
	60	96.16	136.04	39.88
	90	147.43	221.86	74.43
	120	256.94	338.855	81.915
Slope		**2.31**	**3.04**	**0.73**
3-906	30	28.28	47.79	19.51
	60	59.35	107.01	47.66
	90	93.80	202.53	108.73
	120	125.26	283.92	158.66
Slope		**1.08**	**2.68**	**1.60**
3-907	30	39.11	56.03	27.75
	60	65.85	82.49	23.14
	90	137.77	243.25	149.45
	120	191.84	392.98	267.72
Slope		**1.77**	**3.91**	**2.82**
3-908				
	75	131.41	101.51	-29.90
	90	168.49	129.70	-38.80
	120	220.85	201.74	-19.11
Slope		**1.95**	**2.25**	**0.30**
Group Slope Mean				
Mean		**1.78**	**2.97**	**1.36**
SEM		**0.26**	**0.35**	**0.56**

Table 4: Migration EM Data - Young					
		Control		CM	
	Time (min)	EM Mean	SEM	EM Mean	SEM
EM Mean	30	36.68	4.33	55.80	4.56
	60	73.79	11.34	108.51	15.48
	90	136.87	15.72	222.55	11.76
	120	198.72	27.87	338.59	31.48
EM Mean: control subtracted	30			22.73	2.54
	60			36.89	7.23
	90			110.87	21.68
	120			169.43	53.91

Table 5: Migration - Old				
Animal	**Time (min)**	**2% FBS DMEM**	**Conc. CM**	**-Control**
O807	30	31.22	38.1	6.88
	60	61.56	52.07	-9.49
	90	100.92	84.85	-16.07
	120	159.26	107.02	-52.24
Slope		**1.41**	**0.80**	**-0.61**
O808	30	38.41	40.80	2.38
	60	54.43	52.42	-2.01
	90	82.15	78.38	-3.77
	120	112.90	106.78	-6.12
Slope		**0.84**	**0.75**	**-0.09**
O810	30	43.23	45.67	2.44
	60	57.15	59.10	1.95
	90	100.37	86.65	-13.72
	120	134.82	150.63	15.81
Slope		**1.06**	**1.14**	**0.08**
O911				
	75	53.82	72.63	18.81
	90	71.03	107.61	36.58
	120	103.64	143.55	39.90
Slope		**1.10**	**1.52**	**0.42**
Group Slope Mean				
Mean		**1.10**	**1.05**	**-0.05**
SEM		**0.17**	**0.18**	**0.21**

Table 6: Migration EM Data - Old					
	Time	Control		CM	
		EM Mean	SEM	EM Mean	SEM
EM Mean	30	37.62	3.49	41.52	2.22
	60	57.71	2.08	54.53	2.29
	90	88.62	7.31	89.37	6.33
	120	127.65	12.40	126.99	11.69
EM Mean: control subtracted	30			3.90	1.49
	60			-3.18	3.35
	90			0.75	12.24
	120			-0.66	19.59

Appendix 5: Media Formulations

ATCC Dulbecco's Modified Eagle's Medium (DMEM)

Catalog No. 30-2002

Inorganic Salts (g/liter)

CaCl2 (anhydrous) 0.20000
Fe(NO3)3·9H2O 0.00010
MgSO4 (anhydrous) 0.09770
KCl 0.40000
NaHCO3 1.50000
NaCl 6.40000
NaH2PO4·H2O 0.12500

Amino Acids (g/liter)

L-Arginine·HCl 0.08400
L-Cystine·2HCl 0.06260
L-Glutamine 0.58400
Glycine 0.03000
L-Histidine·HCl·H2O 0.04200
L-Isoleucine 0.10500
L-Leucine 0.10500
L-Lysine·HCl 0.14600
L-Methionine 0.03000
L-Phenylalanine 0.06600
L-Serine 0.04200
L-Threonine 0.09500
L-Tryptophan 0.01600
L-Tyrosine·2Na·2H2O 0.10379
L-Valine 0.09400

Vitamins (g/liter)

Choline Chloride 0.00400
Folic Acid 0.00400
myo-Inositol 0.00720
Nicotinamide 0.00400
D-Pantothenic Acid 0.00400
(hemicalcium)
Pyridoxine·HCl 0.00400
Riboflavin 0.00040
Thiamine·HCl 0.00400

Other (g/liter)

D-Glucose 4.50000
Phenol Red, Sodium Salt 0.01500
Sodium Pyruvate

HyClone Ham's Nutrient Mixture F-10 - Liquid Medium

Catalog Number: SH30025

Inorganic Salts (mg/L)

CaCl2 (anhydrous) 33.2900
CuSO4·5H2O 0.0025
FeSO4·7H2O 0.8340
KCl 285.0000
KH2PO4 83.0000
MgSO4 (anhydrous) 76.6400
NaCl 7400.0000
NaH2PO4 (anhydrous) 153.5800
ZnSO4·7H2O 0.0288

Amino Acids (mg/L)

L-Alanine 8.9100
L-Arginine HCl 211.0000
L-Asparagine H2O 15.0000
L-Aspartic Acid 13.3000
L-Cysteine HCI·H2O 25.0000
L-Glutamic Acid 14.7000
L-Glutamine 146.2000
Glycine 7.5100
L-Histidine HCI·H2O 23.0000
L-Isoleucine 2.6000
L-Leucine 13.1000
L-Lysine HCl 29.3000
L-Methionine 4.4800
L-Phenylalanine 4.9600
L-Proline 11.5000
L-Serine 10.5000
L-Threonine 3.5700
L-Tryptophan 0.6000
L-Tryosine 2Na·2H2O 2.6100
L-Valine 3.5000

Vitamins (mg/L)

d-Biotin 0.0240
D-Ca Pantothenate 0.7150
Choline Chloride 0.6980
Folic Acid 1.3200
Hypoxanthine 2Na 5.4000
Myo-Inositol 0.5410
Niacinamide 0.6150
Pyridoxine HCl 0.2060
Riboflavin 0.3760
Thiamine HCl 1.0120
Thymidine 0.7270
Vitamin B12 1.3600
Lipoic Acid 0.2000

Other (mg/L)

D-Glucose 1100.0000
Phenol Red (Sodium) 1.3000
Sodium Pyruvate 110.0000
NaHCO3 1200.0000

Appendix 6: Abbreviations

ACTC – Adrenocorticotrophin

APC – Antigen Presenting Cell

BM – Basal Media

BrdU – 5'-bromo -2'-deoxyuridine

BSA – Bovine Serum Albumin

CM – Conditioned Media

CME – Crushed Muscle Extract

Con A – Concanavalin A

DMD – Duchene Muscular Dystrophy

DMEM – Dulbecco's Modified Eagle Media

DPBS – Dulbecco's Phosphate Buffered Saline

ECM – Extracellular Matrix

EGF – Epidermal Growth Factor

E-selectin-1 - Endothelial Leukocyte Adhesion Molecule-1

FAL – Femoral Artery Ligation

FBS – Fetal Bovine Serum

FGF – Fibroblast Growth Factor

FGF-R – Fibroblast Growth Factor Receptor

GM – Growth Media

HCl – Hydrochloric Acid

HGF – Hepatocyte Growth Factor

ICAM-1 – Intracellular Adhesion Molecule-1

IGF-1 – Insulin-like Growth Factor-1

IFNγ – Interferon-γ

IL – Interleukin

IL-2 – IL-2 Receptor

ION – Ionomycin

LIF – Leukemia Inhibitory Factor

MAP – Mitogen-activated Protein

M-CSF – Macrophage Colony Stimulating Factor

MHC – Major Histocompatibility Complex

MRF – Myogenic Regulatory Factor

MTJ – Myotendinous Junction

MPC – Muscle Precursor Cell

NK Cells – Natural Killer Cells

NO – Nitric Oxide

NSAID – Non-steroidal anti-inflammatory drugs

PDGF – Platelet-derived Growth Factor

PHA – Phytohemagglutinin

PI-3K – Phosphatidylinositol-3-OH

PMA – Phorbol 12-myristate 13-acetate

PML – Polymorphonuclear Leukocytes

RBC – Red Blood Cell; Erythrocyte

TCR – T Cell Receptor

Tf – Transferrin

TGFβ – Transforming Growth Factor β

TN-C – Tenacin-C

TNF – Tumor Necrosis Factor

TPA – Tetradeanoylphorbol-13-acetate

VCAM-1 – Vascular Cell Adhesion Molecule-1

WBC – White Blood Cell, Leukocyte

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