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The Role of T Cells in Muscle Damage Protective Adaptation

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Doctor of Philosophy

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ABSTRACT

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Skeletal muscle is prone to damage from a range of stimuli. The muscle repair process that ensues is complex, involving several phases and requiring the participation of many different cell types. Among the cells involved are various immune cells including neutrophils, macrophages, monocytes, and eosinophils. More recently, T cells were added to this list of immune cells known to participate in effective muscle repair from traumatic injuries in mice. We recently published data showing that T cells also accumulate in human muscle following contraction-induced damage. These data suggested that T cells might be involved an adaptation known as the repeated bout effect that renders muscle protected from future damage after an initial exposure. This document contains research on the role of the immune system, particularly T cells, in the "repeated bout effect."

Key Words: inflammation, exercise-induced muscle damage, repeated bout effect, flow cytometry, T lymphocyte, lengthening contraction, eccentric contraction

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INTRODUCTION

Skeletal muscle injury can be sustained in many ways, including from automobile accidents, lacerations, surgeries, toxin exposure, blood flow obstruction, and exercise (Gehrig and Lynch, 2011). These are among the most commonly reported medical incidents, affecting 107 million adults (Decade, 2008). In parallel with their common occurrence comes a significant financial burden, estimated to cost $849 billion annually in the US (Decade, 2008). No drug targeted to facilitate muscle regeneration is in use (Gehrig and Lynch, 2011). Therefore, more effective interventions that improve muscle regeneration outcomes following injury could alleviate much personal suffering and economic burden. Gaps in our understanding of the complex processes that govern muscle repair impede the development of such interventions.

Due to the complexity of living systems, guiding intrinsic body systems that are already in place to fight disease is a sensible therapeutic approach. For example, a promising therapy for fighting cancer involves altering the patient's T cells so they can readily recognize and kill cancer cells (Dai et al., 2016). In skeletal muscle, there exists an intrinsic damage-protective phenomenon called the repeated bout effect (RBE). The RBE is commonly observed following a novel exercise bout, where muscle damage (e.g., force loss, soreness) is attenuated when the exercise is repeated in the days to weeks following the initial bout. The adaptations that occur in the interim, which render the muscle subsequently resistant to damage, are not fully understood. Studies show that changes in nerve activation, muscle mechanical properties, the extracellular matrix, and inflammation may play a role in RBE (Hyldahl et al., 2017). However, significant gaps remain in our understanding of the mechanisms of RBE. An improved understanding of RBE may be exploited to develop much needed treatment strategies for muscle injuries.

An unexplored possibility is that the immune system may drive RBE in a way akin to its well-established role in protecting against repeated infection. The adaptive immune system, namely T and B cells, are capable of generating memory against antigens of foreign pathogens such that upon secondary exposure a larger and more rapid immune response is mounted, which clears the infection more effectively. Recent studies show that in addition to their classic immunological functions, T cells are also involved in muscle repair from severe muscle injury (Burzyn et al., 2013;Zhang et al., 2014;Castiglioni et al., 2015). Moreover, one study (Burzyn et al., 2013) showed that the main population of T cells that participates in muscle repair is different from T cells found elsewhere in the body in that they harbor characteristic T cell receptor gene arrangements and express a distinct transcriptomic repertoire. This suggests that these cells are generated specifically for the purpose of facilitating muscle repair. A recent study from our lab (Deyhle et al., 2015) showed that T cells are present in human muscle following exercise-induced muscle damage. Most interesting, however, was that there were significantly more T cells in the muscles following a second bout of damaging contractions four weeks later. These data suggest that T cells may have become sensitized to the muscle damage after the first bout of exercise-induced muscle damage and were then primed to respond more powerfully in response to a repeated exposure to exercise-induced muscle damage. In other words, just as T cells develop memory of a specific pathogen so they may more effectively clear it upon reexposure, they may likewise develop "memory" of muscle damage and more effectively facilitate muscle repair upon reinjury. The mechanism for how this may occur is currently unknown.

The purpose of this dissertation project is to test the hypotheses that T cells are instrumental in the RBE in the same way that they protect against repeated infection. Study I

provides a broad assessment of intramuscular inflammation following a single and repeated bout of damaging exercise in humans. Study II provides a time course characterization of T cell accumulation in muscle following damaging contractions. Study III provides a detailed characterization of intramuscular T cell accumulation using flow cytometry following single and repeated bouts of damaging muscle contractions. Additionally, Study III tests the hypothesis that T cells mediate the RBE by carrying out an adoptive cell transfer study.

AIMS AND HYPOTHESES

Study I Skeletal Muscle Inflammation Following Repeated Bouts of Lengthening Contractions in Humans

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Statement of the Problem

The mechanisms underlying the repeated bout effect (RBE) are not fully understood. To gain better inflammation-related mechanistic insight into the RBE, this study used a cytokine screening assay to probe for potential inflammation-related molecular and cellular mediators of the RBE.

Specific Aim 1

Use a 29-plex cytokine multiplexing assay to obtain a broad picture of the muscle inflammatory environment 1 day before and 2 days after an initial and a repeated bout of muscle-damaging contractions.

Hypothesis 1

Proinflammatory (Th1) cytokines and chemokines will be upregulated following the initial bout, and will be relatively attenuated following a repeated bout.

Brief Methods

Seven men (23 ± 2.1 years old) and seven women (25.6 ± 2.5 years old) underwent two bouts of muscle-damaging contractions. 28 days separated bout 1 (B1) and bout 2 (B2). A total of four muscle biopsy samples were taken from the vastus lateralis muscle at the following time points; 1 day before B1, 2 days after B1, 1 day before B2, and 2 days after B2. Using a multiplexing fluorescent imager (Luminex Magpix), the protein concentrations of 29 inflammation-related cytokines were quantified from muscle biopsy homogenates.

Brief Results

Only about one-third of the assayed cytokines were detectable in the muscle samples. Of those detected, only three cytokines changed significantly. Contrary to our hypothesis, no significant increase in proinflammatory cytokines was observed 2 days following the initial bout. Also contrary to our hypothesis, the pro-inflammatory Th1 chemokines CCL2 (also called MCP-1) and CXCL10 (also called IP-10) increased 2 days after the second bout of damaging contractions. In keeping with increased Th1 inflammation following a repeated bout, the Th2 cytokine IL-4 was attenuated following the repeated bout. CCL2 and CXCL10 are important chemokines of inflammatory monocytes/ macrophages and Th1-polarized T cells, respectively. Thus, the increase in CCL2 and CXCL10 implicate the involvement of these immune cell types in muscle repair from contraction-induced damage.

Specific Aim 2

Measure the accumulation of pro-inflammatory macrophages (CD68+) and T cells (CD8+) in the muscle biopsy samples, as guided by the finding that the chemokines CCL2 and CXCL10 increased in the muscle.

Hypothesis 2

Macrophage and T cell concentrations will parallel the changes in their respective chemokines, such that they will moderately increase following the B1 but will increase to a greater degree following B2.

Brief Methods

Muscle cross sections were mounted on microscope slides and stained with DAPI and fluorochrome-conjugated antibodies against dystrophin and either CD8 (to assay for T cells) or CD68 to assay for macrophages. Data were expressed as CD8+ and CD68+ cells per imaged myofiber.

Brief Results

Consistent with hypothesis 2, both macrophage and T cell numbers in muscle correlated ($p < 0.05$) with their respective chemokines, with the greatest numbers observed 48 hours after B2. Macrophages were significantly increased at 48 hours post-B2 (13 ± 7 cells/100 fibers) compared to pre-B1 (9 ± 4 cells/100 fibers). CD8 T cells were significantly increased at 48 hours post-B2 (27 ± 28 cells/100 fibers) compared to pre-B1 (5 ± 4 cells/100 fibers).

Study II A Time Course Characterization of Muscle T Cell Accumulation Following Damaging Contractions in Humans

Statement of the Problem

To date, only two studies have reported T cell accumulation in human muscle following damaging contractions, and both studies have only collected data at a single time point following the damaging stimulus. We (Deyhle et al., 2015) observed T cells in muscle at 48 hours after, while Marklund (Marklund et al., 2013) measured muscle T cell accumulation at 24 hours after intense exercise. Thus, there is very limited information as to the time course of appearance and disappearance of T cells in muscle following damaging contractions in human.

Specific Aim 1

To characterize the time course of T cell accumulation in human muscle following damaging contractions.

Hypothesis 1

T cells will peak at 24 hours after a damaging stimulus and begin to subside by 72 hours after.

Brief Methods

Muscle biopsy samples were obtained from the vastus lateralis from six men and three women (22.2 ± 2.4 years of age) before and at 3 hours, 24 hours and 72 hours after a bout of 300 maximal-effort lengthening contractions of the knee extensor muscles. Muscle cross sections were mounted on microscope slides and stained with DAPI and fluorochrome-conjugated antibodies against CD8 and dystrophin. CD8+ cells were counted using fluorescence microscopy.

Brief Results

CD8+ T cell concentrations were statistically similar to predamage levels at 3h and 24 hours after damaging contractions, but greatly increased compared to predamage (5 fold, $p < 0.05$) at 72 hours. An interesting and unexpected finding was that females displayed greater T cell accumulation following damage than males did (Sex X Time, $p < 0.05$). These data indicate that T cell accumulation in muscle following exercise-induced damage peaks around 3 days or later. These data are consistent with literature showing that T cell accumulation peaks at 3 or 4 days following myotoxic injury in rodents (Burzyn et al., 2013;Castiglioni et al., 2015).

Study III The Role of T cells in the Repeated Bout Effect

Statement of the Problem

T cells are known to play essential roles in muscle repair from traumatic injury. It is not known whether T cells are needed for muscle repair or adaptation from contraction-induced damage.

Specific Aim 1

Establish a model of the repeated RBE and the contralateral RBE (cRBE) in rats using electrically stimulated lengthening contractions to induce damage.

Hypothesis 1

An initial bout of lengthening contractions will cause more damage than a repeated bout of lengthening contractions when the repeated bout is done on the damage-experienced muscle or the contralateral damage-naïve muscle

Brief Methods

Male Lewis Rats were subjected to unilateral in vivo lengthening contractions of the anterior crural muscle group to cause muscle damage. One group of rats did a single bout of lengthening contractions (SB3D). Another group completed two identical bouts separated by 2 weeks on the same limb (RB). A third group also did two bouts of lengthening contractions separated by 2 weeks with the second bout on the contralateral limb (cRB). Muscle strength was measured before, 5 minutes after, and 3 days after each bout of LC. Each group of rats was euthanized immediately after their last strength test. Muscle damage was assessed by the degree of force loss and restoration and the frequency of damaged/necrotic muscle fibers.

Brief Results

Muscle strength was restored by 3 days post-LC in the RB group but no recovery was observed in the cRB group. The frequency of damaged fibers was greatly reduced in the RB group, but there was no apparent reduction in the cRB group. On the basis of both muscle function and histological evidence of damage, the RBE was observed, but there was no evidence of the cRBE.

Specific Aim 2

Compare the quality and quantity of T cells in muscle following a single and a repeated bout of damaging contractions.

Hypothesis 2a

Both CD8 and CD4 T cells will increase in damaged muscles compared to the nondamaged muscle, and a larger percentage of CD4 T cells in the muscle will be regulatory T cells (Tregs) compared to those in the spleen.

Hypothesis 2b

The magnitude of T cell infiltration will be greater, and a larger percentage of T cells will express cell surface markers indicative of a memory phenotype (CD127+) after a repeated bout compared to after a single bout of muscle damage.

Hypothesis 2c

A more robust accumulation of memory T cells will also occur following damage to contralateral muscle (not previously damaged) if the opposite limb had previously been damaged.

Brief Methods

The spleens and part of the damaged and control muscle samples from the groups of rats described above were prepared for multicolor flow cytometry. We probed for 6 antigens for the purpose of characterizing the muscle T cell infiltrate. Antigens: CD45, CD3, CD4, CD8α, CD127 and Foxp3

Brief Results

CD4 and CD8 T cells increased substantially in the damaged muscles compared to the levels seen in undamaged muscles. Contrary to our hypothesis, Tregs (Foxp3+, CD4+ T cells) were not enriched in the muscle compared to the spleen, rather their percentage contribution was similar. Also not anticipated, T cell content was substantially blunted in the repeated bout rats. The T cell accumulation in the contralateral repeated bout rats was not different to what was seen in the rats that did a single bout. The percentage of muscle T cells expressing the memory marker (CD127) was quite low and was not different among conditions.

Specific Aim 3

Test if T cells contribute to the damage-protected phenotype of the RBE and cRBE.

Hypothesis 3

Rats receiving adoptive T cell transfer from damage-experienced donor rats will sustain less muscle damage following lengthening contractions compared to rats receiving T cells from damage-naïve donor rats.

Brief Methods

T cells were harvested and sorted (MACS) from the spleens and inguinal lymph node from two donor rats. One donor rat (damage-experienced donor) did a bout of LC, as described above two weeks prior to tissue harvesting. The other donor (damage-naïve) underwent no

muscle contraction procedure prior to tissue harvesting. T cells (2 x 10^6) were injected into the tail vein of the recipient rats (n = 5 per donor) immediately after a bout of LC. The recipient rats were euthanized 3 days later. Muscle damage was assessed based on the loss and restoration of muscle strength, and the frequency of damaged/necrotic muscle fibers in the tibialis anterior muscle.

Brief Results

No differences were found in muscle damage between the two groups of recipient rats.

MANUSCRIPTS

Study I Skeletal Muscle Inflammation Following Repeated Bouts of Lengthening Contractions in Humans

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Abstract

Skeletal muscle responds to exercise-induced damage by orchestrating an adaptive process that protects the muscle from damage by subsequent bouts of exercise, a phenomenon called the repeated bout effect (RBE). The mechanisms underlying the RBE are not understood. We hypothesized that an attenuated inflammation response following a repeated bout of lengthening contractions (LC) would be coincidental with a RBE, suggesting a potential relationship. Fourteen men (n = 7) and women (n = 7) completed 2 bouts of lengthening contractions (LC) separated by 28 days. Muscle biopsies were taken before the first bout (B1) from the nonexercised leg, and from the exercised leg 2 days and 27 days post-B1 and 2 days following the second bout (B2). A 29-plex cytokine array identified alterations in inflammatory cytokines. Immunohistochemistry quantified inflammatory cell infiltration and major histocompatibility complex class 1 (MHC-I). Muscle soreness was attenuated in the days

following B2 relative to B1, indicating a RBE. Intramuscular monocyte chemoattractant protein (MCP1) and interferon gamma-induced protein 10 (IP10) increased following B2 relative to the preexercise sample (7 pg/ml to 52 pg/ml, and 11 pg/ml to 36 pg/ml, respectively $p < 0.05$). Interleukin 4 (IL4) decreased (26 pg/ml to 13 pg/ml, $p < 0.05$) following B2 relative to the preexercise sample. Infiltration of CD68+ macrophages and CD8+ T cells were evident following B2, but not B1. Moreover, CD8+ T cells were observed infiltrating apparently necrotic muscle fibers. No changes in MHC-I were found. We conclude that inflammation is not attenuated following a repeated bout of LC and that CD8+ T cells may play a role in muscle adaptation following LC. Moreover, it appears that the muscle or the immune system becomes sensitized to an initial bout of damaging exercise such that inflammatory cell infiltration into the muscle is enhanced upon a repeated bout of damaging exercise.

Introduction

A truly remarkable property of skeletal muscle is that it maintains an intrinsic protective mechanism, whereby it swiftly adapts following exercise-induced damage, making it capable of resisting future damage. This phenomenon has been recognized for over half a century (Highman and Altland, 1963) and is commonly referred to in the literature as the repeated bout effect (RBE) (Nosaka and Clarkson, 1995). Over the years, a significant body of literature has been developed to describe the parameters of an original stimulus necessary to induce the protective effect (Lavender and Nosaka, 2008;Barroso et al., 2010;Muthalib et al., 2011). Nevertheless, the molecular underpinnings and deeper mechanisms of the RBE have been relatively under studied and are not well known. A more complete understanding of the mechanisms that mediate the RBE adaptation(s) may have significant clinical value with vast applications, from the

prevention and management of muscle related injuries, to the treatment of muscle degenerative disorders.

Potential mechanisms for the RBE that have been explored recently by our laboratory and others have implicated extracellular matrix remodeling (Hyldahl et al., 2015), and muscle architectural alterations (Lau et al., 2015). An older hypothesis, supported initially by the results of animal studies, proposes that reduced inflammation following a second damage exposure reduces secondary damage and subsequent markers of muscle damage (McHugh, 2003). Indeed, rodent studies have shown a blunted inflammatory response in conjunction with muscle damage markers when lengthening contractions (LC) were preceded by either a bout of passive stretches, or a prior bout of LC (Pizza et al., 2002;Koh et al., 2003). However, inflammation has not been comprehensively assessed following repeated bouts of damaging exercise in humans, leaving doubt as to whether this mechanism is important in mediating the protective effect in human skeletal muscle. Moreover, few studies have examined the intramuscular inflammatory cytokine environment following repeated bouts of damaging exercise. Information from these studies is also somewhat limiting given that the cytokines were measured only at the mRNA level (Hubal et al., 2008;Mackey et al., 2011).

Thus, the purpose of this study was to broadly assess markers of inflammation (i.e., cytokines and infiltrating lymphoid and myeloid cells) in human muscle in a well-established paradigm of the repeated bout effect in the knee extensor muscles. Overall, we hypothesized that muscle inflammatory markers would be present following the first bout of LC, as reported by others (Paulsen et al., 2012). Furthermore, we expected that these same markers would be attenuated following a repeated bout of LC, consistent with the attenuation of other direct and indirect markers of muscle damage.

Methods

Subjects

Fourteen young, healthy, men (n = 7; age 23.3 ± 2.1) and women (n = 7; age 25.6 ± 2.5) volunteered to participate in this study. The activity level of the subjects ranged from moderately active to sedentary. The subjects had not participated in weight training or consistent, structured physical activity in the past six months. As a participant in the study, the subjects agreed to not change their activity levels or use any form of analgesic or nonsteroidal anti-inflammatory drugs during the duration of the study. All subjects signed a written informed consent document authorized by the Brigham Young University Institutional Review Board and were informed of the procedures as well as potential risks.

Study Design

This study consisted of 2 bouts of LC spaced 28 days apart. 28 days between bouts was chosen because it allows for sufficient washout of acute symptoms of the initial bout (e.g., soreness, strength loss, serum enzymes) and is still within the 6- to 9-month window of time that the repeated bout effect lasts after an initial bout (Nosaka et al., 2001). Each bout of exercise was performed on the same randomly selected leg. Four muscle biopsies were taken. The first biopsy was taken 1 day before the first bout of exercise (designated as day -1) from the nonexercised leg. Two days after the first bout (B1) of exercise, the second biopsy was taken (designated as 2 days post-B1) from the exercised leg. The third biopsy (designated as 27 days post-B1) was taken from the same exercised leg 27 days after the B1 (also described as the day before the second bout of exercise). The next day (28 days after B1) the subjects completed the second bout of LC (B2) using the same exercise leg, and a muscle biopsy was taken 2 days later on the exercised leg (designated as 2 days post-B2). The post-LC biopsy was taken at 48 hours because

this is around the time when many markers of muscle damage are greatest (strength loss, soreness, plasma creatine kinase activity). Muscle soreness was assessed 1 day prior to the exercise and then again 2, 3, 4, and 5 days following each bout of LC. Throughout the duration of the study, subjects were instructed to maintain their regular diet and level of physical activity, as well as to refrain from use of caffeine, alcohol and NSAIDs. One subject reported using ice 2 days after the first bout of exercise to reduce muscle soreness.

Exercise Protocol

The exercise sessions each included 30 sets of 10 maximal eccentric contractions. The exercises were performed on an isokinetic dynamometer (Biodex Medical Systems, Shirley, NY, USA). Subjects were instructed to resist the lever arm from 30° (0° = full extension) of flexion to the subject's maximal knee flexion ROM (approximately 110°), moving at an angular velocity of $120° \cdot s^{-1}$. Between each set, subjects were given a 1-minute rest. This same exercise protocol has been widely used to induce muscle damage and a repeated bout effect (McKay et al., 2010;Hyldahl et al., 2015). There were no differences in the amount of work performed for each bout of exercise.

Muscle Biopsy Procedure

Four total muscle biopsies were taken from the *m. vastus lateralis*. This muscle was chosen because it is easy to access and no major blood vessels or nerves are in the area. After using 2% lidocaine as a local anesthetic, a small incision was made past skin and fascia. Percutaneous muscle biopsies were then collected through a manual suction method. The first biopsy incision was made approximately 15 cm proximal to the insertion of the *vastus lateralis* of the nonexercised leg. Subsequent biopsies from the exercised leg were taken approximately 3-5 cm proximal to the previous biopsy to minimize the effects of previous biopsies. Care was also

taken to angle the insertion of the needle away from the previous biopsy to minimize effects of previous biopsies (Van Thienen et al., 2014). Muscle samples were preserved for both immunohisochemisty and protein analysis. Samples for microscopic analysis were mounted on a cork and frozen in isopentane-cooled liquid nitrogen while samples used for protein homogenates were snap frozen in liquid nitrogen. All samples were stored at −80ºC.

Soreness Assessments

Perceived muscle soreness was assessed using a visual analog scale (VAS). The VAS consisted of a 100 mm line with anchors indicating "no pain" on one end and "unbearable pain" on the opposite end. Subjects were instructed to perform 2 body weight squats and then immediately evaluate soreness by marking on the VAS scale with a vertical line. The distance from the no pain end of the VAS was measured and used for analysis.

Immunohistochemistry

Muscle biopsy samples used for histochemical staining were cut into 8 µm sections using a cryostat at −25°C. Cut samples were mounted on Superfrost slides and allowed to air dry for 10 minutes. Sections stained for CD8 were fixed in 2% paraformaldehyde for 10 minutes. After fixation, samples were washed in phosphate-buffered saline (PBS) and then incubated in primary antibodies overnight at 4°C (CD8 diluted 1:50 in PBS and dystrophin 1:500). On the following day, samples were washed and incubated in secondary antibodies diluted 1:100 in PBS for 30 minutes at 37°C. DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride) was added to the secondary antibody solution to visualize nuclei. Following incubation, samples were washed in PBS and dipped in water. The slides were then dried and mounted with Fluoroshield histology mounting medium (Sigma-Aldrich, St. Louis, MO, USA). Sections stained for CD68 were fixed in 2% paraformaldehyde for 4 to 8 minutes. Following fixation, sections were permeabilized in

0.2% Triton X-100 for 10 minutes and then blocked in a 2% bovine serum albumin (BSA), 5% fetal bovine serum (FBS) solution for 30 minutes at room temperature. Sections were incubated in the primary antibodies (CD68 and dystrophin diluted in blocking cocktail at 1:300 and 1:200, respectively) in a humidified chamber overnight at 4°C. Following several washes, sections were then incubated in the appropriate secondary antibodies (both diluted at 1:200 in PBS) for 30 minutes at 37°C. Following multiple washes, slides were incubated in DAPI for 30 minutes at 37°C. Stained slides were washed in PBST (PBS tween-20), dried, then mounted using Fluoroshield histology mounting medium. Sections stained for MHC-I were fixed in 100% ice-cold acetone for 10 minutes. Following a rinse with PBS, the samples were placed in a humidified chamber and were incubated in blocking solution for 1 hour (5% horse serum and 0.2% Trition X 100). Following blocking, the samples were washed in PBS. The samples were then incubated with the primary antibody diluted 1:100 in PBS with 2% bovine serum albumin (BSA) in a humidified chamber for 1 hour at room temperature. The wash procedure was repeated and the samples were incubated in a secondary antibody diluted 1:100 in PBS with DAPI. All slides were imaged on an Olympus IX73 fluorescence-capable inverted microscope. Antibody stain validity was verified using secondary-only controls. Primary antibodies used were CD8, mouse monoclonal IS623 (Dako, Glostrup, Denmark); CD68, mouse monoclonal M0718 (Dako, Glostrup, Denmark); dystrophin, rabbit polyclonal (ab15277; Abcam, Cambridge, United Kingdom) and HLA-ABC (MHC-I) mouse monoclonal M0736 (Dako, Glostrup, Denmark). Secondary antibodies used Alexa Fluor 488 goat anti-rabbit A11029 (Life Technologies, Carlsbad, CA, USA); Cy3 anti-rabbit 115-165-003 (Jackson ImmunoResearch Laboratories, West Grove, PA).

Quantification of Immunofluorescent Images

Quantification of immunofluorescent images was carried out by investigators that were blind to both condition and time point. For CD68 analyses, enumeration was accomplished by analyzing 10 randomly acquired fields using a 40X objective. A cell was considered CD68+ when the green fluorescent stain clearly surrounded a DAPI+ nucleus (see arrows in Figure 4). An average of 210 ± 10 muscle fibers were analyzed per subject per time point. Because CD8+ cells appeared to be much less frequent than CD68+ cells, CD8 enumeration was made by imaging the entire section, using a 20X objective. This resulted in approximately 10-20 acquired fields per time point, depending on the size of the muscle cross section. An average of 801.9 ± 329.3 muscle fibers were analyzed per subject per time point. To assess MHC-I content, the entire muscle section was imaged at 10 X magnification. Two to five images per time point were acquired to capture the entire section. Images were taken using the same exposure and gain settings within subjects. For quantification, the total MHC-I immunoreactive area was expressed relative to the total area of the imaged section.

Cytokine Magnetic Bead Multiplex

The frozen muscle samples were homogenized with a Total Protein Extraction Kit (Millipore, Billerica, MA) with protease and phosphatase inhibitor cocktails (Thermo Scientific, Rockford, IL). A Direct Detect Spectrometer (Millipore, Billerica, MA) was used to determine the total protein concentrations. The Luminex Magpix multiplexing platform was then used for multianalyte profiling of biopsy sample homogenates. Cytokines in muscle homogenates were measured using a 29-plex cytokine kit in compliance with manufacturer's parameters (EDM Millipore, Billerica, MA). Briefly, 25 µg of protein homogenate were incubated overnight at 4°C with antibody-conjugated magnetic beads. The bead-complex was then washed, followed by a

30-minute incubation at RT on a plate shaker in biotinylated detection antibody. Streptavidin-phycoerythrin was subsequently added and samples were incubated for an additional for 30 minutes on a plate shaker at RT. A Magpix (Luminex Corporation, Austin, TX) system was used to quantify bead-complexes. Data analysis was based on a minimum of 80 beads using median fluorescence values.

Statistics

Perceived muscle soreness was evaluated using the area under the curve (AUC) for soreness response for the days after B1 and B2 for each subject. AUC measurements one day before exercise and days 2 through 5 after exercise were used to calculate AUC for each bout. A paired t-test was used to test for differences in AUC between bouts. A one-way repeated measures analysis of variance (ANOVA) was used to test for differences in immunohistochemical data. Each individual cytokine of interest was also analyzed using one-way repeated measures ANOVAs. A Tukey-Kramer HSD test was used for pairwise comparisons when the F-statistic revealed a significant p-value. Correlation analyses were carried out using a mixed models linear regression technique with blocking on subjects. Blocking on subjects was used to account for the lack of independence in the data. Correlation analysis was only investigated when a relationship between variables was anticipated. To normalize distributions and homogenize variance, the following variables were log transformed; IL-4, MCP-1, IP-10, soreness. Prism Graphpad (V6.0b; San Diego, CA, USA) was used for AUC and t-test calculations for soreness data. JMP® Pro (V11.2; SAS Institute, Cary, NC, USA) was used for all other analyses. Results were considered statistically significant at $p < 0.05$.

Results

Soreness

Changes in the overall magnitude of delayed onset muscle soreness associated with B1 and B2 were compared to verify the presence of a repeated bout effect. Muscle soreness in the knee extensor muscle group increased after B1 and B2 and returned to baseline values 5 days after exercise for both bouts (Figure 1.1A). Muscle soreness peaked 2 days after exercise for B1 and B2. To quantify differences in overall soreness response in the days after each bout, we compared the AUC of soreness for B1 and B2. AUC of soreness was significantly reduced at B2 compared to B1 (33% reduction, ± 11%, one-tailed $p = 0.032$) (Figure 1.1B).

Cytokine Array

To determine the extent of inflammation and identify potentially important inflammatory cytokines that may mediate the repeated bout effect, we performed an unbiased 29-plex cytokine screen. Among the twenty-nine cytokines measured, only monocyte chemoattractant protein 1 (MCP-1 also known as CCl2), Interleukin 4 (IL-4) and Interferon-gamma-inducible protein 10 (IP-10 also known as CXCL10) showed a significant main effect among biopsy time points ($p < 0.05$). Figure 1.2 shows comparisons of the mean values of IP-10, MCP-1, and IL-4 across sampling time points. IP-10 was significantly increased at post-B2 relative to pre-B1 (2.26 ± 1.31 fold, $p = 0.021$). Potential trending differences may be present between pre-B1 and post-B1 ($p = 0.11$) and pre-B2 and post-B2 ($p = 0.12$). MCP-1 content at post-B2 was significantly elevated compared to pre-B1 (2.97 ± 1.35 fold, $p = 0.0032$). MCP-1 content at pre-B2 showed a suggestive but inconclusive increase over pre-B1 (2.15 ± 1.35 fold $p = 0.0603$). In contrast, IL-4 showed a significant decrease from pre-B1 to post-B2 (3.82 ± 1.44 fold, $p = 0.041$) and from pre-B1 to pre-B2 (3.72 ± 1.44 fold, $p = 0.005$). An inconclusive decreasing trend in IL-4 content was

also observed between pre-B2 and post-B2 ($p = 0.14$). Results of all other nonsignificant detectable cytokines can be found in Table 1.

Macrophage Infiltration

As MCP-1 is an important mediator of macrophage infiltration into damaged skeletal muscle (Shireman et al., 2007), we analyzed the muscle cross sections for the presence of $CD68^+$ macrophages using immunohistochemistry (Figure 1.3A). Given that a significant increase in MCP-1 was only detectable following B2, we expected that macrophage content would be increased post-B2 relative to the pre-B1 measurement. As expected, the number of $CD68^+$ cells per 100 muscle fibers increased by 4.6 ± 1.5 cells per 100 fibers following B2 compared to the pre-B1 measurement ($p = 0.021$) (Figure 1.3B). Additionally, we noted an increasing trend in the number of $CD68^+$ macrophages at post-B1 compared to the pre-B1 measurement ($p = 0.08$).

T Cell Infiltration

Our analysis of cytokines identified increases in few cytokines 48 hours post-LC. Among the differentially altered cytokines, IP-10 was interesting, as we had previously found this cytokine to be increased 24 hours following a single bout of LC (Hyldahl et al., 2014). IP-10 appears to be important in the activation and trafficking of T cells to infected tissues (Khan et al., 2000;Dufour et al., 2002) and is found to be upregulated in the muscle of individuals suffering from inflammatory myopathies accompanied by increased levels of cytotoxic T cells (De Paepe et al., 2005). Guided by our finding of increased levels of IP-10 following B2, we assessed the presence of CD8+ T cells in the muscle samples using an antibody against CD8 (Figures 1.4A and 1.4B). Overall, the occurrence of CD8+ T cells in the muscle samples was infrequent relative to other cell types that have been identified in human skeletal muscle. Nevertheless, CD8+ T cells per 100 muscle fibers increased following B2 (2.7 ± 2.8) relative to pre-B1 (0.5 ± 0.4) ($p =$

0.003) and post-B1 (1.0 ± 0.7) ($p = 0.027$) (Figure 1.4C). CD8+ cells were also found infiltrating what appeared to be regenerating myofibers, as evidenced by diminished and discontinuous dystrophin staining pattern (Figure 1.4B) and in spaces adjacent to what appeared to be vasculature (i.e., capillaries).

Major Histocompatibility Complex Class I (MHC-I)

MHC-I proteins display antigens on the cell surface for CD8+ T cell-mediated surveillance. MHC-I is not normally expressed by mature skeletal muscle cells. However, MHC-I expression can be greatly upregulated in muscle suffering from inflammatory myopathies (Englund et al., 2001;Choi et al., 2009) and in response to extreme exercise (Marklund et al., 2013). Given the relationship between CD8+ T cell and MHC-I, we measured MHC-I content via immunohistochemistry in the muscle to test the hypothesis that MHC-I content would increase in the muscle in parallel with the observed changes in T cell content. Contrary to our hypothesis, we found no significant changes in MHC-I immunoreactivity (Figures 1.5A and 1.5B). MHC-I immunoreactivity was found consistently on capillaries and associated with interstitial mononuclear cells. MHC-I staining was also commonly found around the sarcolemma, though it was rarely found completely surrounding fibers (Figure 1.5A). In one subject (a high responder) evidence of sarcoplasmic MHC-I immunoreactivity was found (Figure 1.5A).

Linear Regression Analyses

Mixed models linear regression analyses were used to determine if the content of the chemotactic cytokines, IP-10 and MCP-1, were related to the number of infiltrating CD8+ T cells and $CD68^+$ macrophages. Figure 1.6 shows graphical representations of each correlation. A strong relationship was found between $\log_e$ MCP-1 and $CD68^+$ cells per muscle fiber ($r^2 = 0.71$,

slope = 0.029 ± 0.007, $p < 0.0001$). A similarly strong relationship was also observed between $\log_e$ IP-10 and $CD68^+$ macrophages per muscle fiber ($r^2 = 0.67$, slope = 0.027 ± 0.008, p = 0.001). Weaker yet significant correlations were found between these cytokines and CD8+ T cells. Log_e IP-10 vs. CD8+ T cells per fiber yielded an r^2 of 0.26 (slope = 0.007 ± 0.009, p = 0.024). MCP-1 vs. CD8+ T cells per fiber yielded an $r^2 = 0.33$ (slope = 0.008 ± 0.003, p = 0.006). No significant correlation between MHC-I immunoreactivity and CD8+ T cell infiltration was found ($r^2 = 0.25$, slope = -0.001 ± 0.002 p = 0.71).

Discussion

Inflammation following damaging exercise has been thought to cause further tissue damage mediated by muscle-invading leukocytes (Toumi and Best, 2003). A hypothesized mechanism of the repeated bout effect posits that inflammation following damaging exercise, and in turn secondary muscle damage, is blunted following a second bout of damaging exercise (McHugh et al., 1999). Overwhelmingly, the data presented here do not support the hypothesis of an attenuated inflammatory response following a second bout of LC. On the contrary, the data suggest an unaltered or slightly enhanced inflammatory response following a second bout of LC. The overall reduction in delayed onset muscle soreness (an established indicator of muscle damage (Clarkson and Hubal, 2002)) in the days following B2 provides evidence of a repeated bout effect. Additionally, a previously published study that reported on the same group of subjects showed reduced strength loss and insignificant increases in serum creatine kinase activity following B2 (Hyldahl et al., 2015).

Surprisingly, only three cytokines showed significant changes among all 29 measured. Inflammatory cytokines MCP-1 and IP-10 increased after B2, while the anti-inflammatory cytokine IL-4 was reduced before and after B2. The increases in cytokine concentration also

mirrored (and were positively correlated with) the changes in macrophage and T cell infiltration, providing suggestive evidence for a chemotactic relationship between these cytokines and inflammatory cell populations in human muscle. Our observation that these events were only increased following B2 suggests that the initial bout of LC may have sensitized the muscle towards a greater, and therefore more persistent, state of inflammation following the second bout of LC. In other words, the muscle seems to "remember" the damaging insult and is sensitized to initiate a more robust recruitment of immune cells in response to a repeated insult, reminiscent of the way the adaptive immune system responds to a repeated antigen exposure. Furthermore, muscle soreness is reduced concurrent with an increase in inflammation, indicating that a positive relationship between these variables is unlikely.

Cytokines

Increases in MCP-1 following muscle damage have been widely reported in the animal and human literature, and its augmentation following damage appears to be necessary for healthy muscle regeneration (Warren et al., 2004;Shireman et al., 2007). Consistent with the findings of Hubal and colleagues (Hubal et al., 2008), we observed a significant increase in MCP-1 post-B2. However, this finding is in contrast with another study that showed an attenuation of MCP-1 gene expression following a repeated bout of damaging electrically stimulated isometric contractions (Mackey et al., 2011). Electrically stimulated contractions and voluntary eccentric contractions have been shown to cause distinct histological and biochemical responses (Crameri et al., 2007). Therefore, the reason for these disparate findings may be related to the differing modes of damage-induction. Furthermore, both of these studies measured MCP-1 at the transcript level, whereas we assessed MCP-1 protein content.

An interesting and novel finding that emerged from the present study is the increase in the classically proinflammatory cytokine IP-10 following B2. IP-10 is an important regulator of T cell trafficking (Dufour et al., 2002;Wang et al., 2015). IP-10 is also involved in inflammation and immune responses in a host of tissues and conditions, yet little data are available regarding the role of IP-10 in skeletal muscle. IP-10 has been identified as a potential therapeutic target for inflammatory myopathies (Crescioli et al., 2012), but is also expressed in unperturbed healthy skeletal muscle (De Paepe et al., 2005). Recent work from our laboratory showed IP-10 to be upregulated in muscle 24 hours after a single bout of lengthening contractions but not shortening contractions (Hyldahl et al., 2014). Like in our previous study, IP-10 concentrations were increased in response to damaging LC, albeit only after a repeated bout (Figure 1.2). Why significant increases were not seen post-B1 might be due to different sampling time points. In the present study, we took muscle samples 48 hours after exercise whereas our previous investigation sampled muscle 24 hours after exercise. Together, these studies suggest IP-10 may play a role in the damage and repair process of muscle adaptation to exercise. More work is needed to characterize the role IP-10 plays in these processes.

IL-4 is an important cytokine in driving the M1 to M2 phenotypic conversion (Villalta et al., 2009). Here we report a decrease in IL-4 protein concentration in muscle tissue from pre-B1 to post-B2 (Figure 1.2). This finding was unexpected because the present hypothesis predicted an attenuated inflammation response around B2, but the observed reduction in IL-4 around B2 is suggestive of an increased proinflammatory state. These data suggest that the increased number of $CD68^{+}$ macrophages after B2 (Figure 1.3B) may have embodied a more proinflammatory phenotype. However, TNF-alpha and IFN-gamma, both promoters of the M1 phenotype (Villalta et al., 2009), were not present in the muscle samples at this time point in detectable quantities

(Table 1). This suggests that the macrophages present post-B2 likely did not display a highly polarized phenotype at either end of the M1/M2 spectrum. Given that macrophages were significantly elevated only at post-B2, when evidence of muscle damage was reduced, suggests that these cells do not aggravate the symptoms (i.e., soreness, force loss and membrane permeability) of exercise-induced damage in healthy human skeletal muscle. This interpretation is consistent with data from Tidball et al. (Tidball et al., 1999), which showed that the loss of sarcolemmal integrity in response to muscle overload was not different in the presence or absence of macrophages. Likewise, another study by the same group showed that macrophages promote muscle repair and regeneration in response to overload-induced damage (Tidball and Wehling-Henricks, 2007).

Inflammatory Cell Infiltration

Consistent with others (Stupka et al., 2001;Mackey et al., 2011) we observed a significant increase in macrophage content in the muscle following damaging exercise, though we are the first, to our knowledge, to report that macrophages accumulate in greater numbers following a second exposure to LC. Another novel finding of the present study is our observation of increased muscle T cell content after a second bout of LC (Figure 1.4C). T cells are known to play a role in inflammatory myopathies (Choi et al., 2009;Crescioli et al., 2012), muscular dystrophies (Spencer et al., 2001;Choi et al., 2009;Madaro and Bouche, 2014) and obesity-related muscle fat accumulation and insulin resistance (Khan et al., 2015). Moreover, the presence of T cells in muscle tissue has been thought to be specific to muscle afflicted with chronic inflammatory pathologies (Madaro and Bouche, 2014). However, one study has demonstrated a significant increase in muscle T cell content following a 24-hour ultraendurance bout (Marklund et al., 2013). The significant increase in T cells observed after B2 in the present

study suggests that these cells may play a role in the adaptive and regenerative processes of healthy human skeletal muscle to damaging exercise. Indeed, recent animal studies show that T cells play a role in muscle regeneration by chemokine and cytokine secretion (Zhang et al., 2014;Fu et al., 2015). A study by Malm et al. (Malm et al., 2000) measured no changes in infiltrating CD8+ T cells in healthy muscle tissue in response to a single bout of damaging eccentric cycling exercise. The authors concluded that T cells do not infiltrate healthy skeletal muscle. Consistent with this study we also did not detect a significant change in T cell content after a single bout of damaging exercise. However, we show that CD8+ T cells do infiltrate healthy muscle, but only do so in appreciable quantities after a repeated bout of damaging exercise. It may be that an initial bout of exercise primes the muscle to more effectively recruit T cells that become manifest at a subsequent bout.

Because no significant changes in MHC-I content was observed, the priming effect may not be mediated by increased muscle MHC-I expression as we had suspected. However, it is possible that damaged muscle may display peptide sequences on MHC-I proteins that are indicative of muscle damage (damage-specific epitope), thereby sensitizing T cells to respond to muscle damage by recognizing muscle damage-specific auto antigens displayed on MHC-I without detectable changes in total MHC-I content. This speculation provides a hypothesized mechanism of the observation that CD8+ T cells increased only after B2. Another possible explanation is that T cells were recruited to the muscle slowly after B1, were insignificantly increased at the 27-day time point (Figure 1.4C), and the higher numbers of T cells that accumulated in the muscle pre-B2 allowed for a more rapid T cell expansion by proliferation, recruitment of more T cells, or both in response to LC of B2. Consistent with this hypothesis, the slightly increased MHC-I content at pre-B2 (statistically insignificant, Figure 1.5B) may play a

role in sensitizing T cells for heightened responsiveness post-B2. It may be that slightly increased MHC-I expression at this time point provided low-affinity subthreshold interactions with MHC-bound nondamage-indicative peptides and the T cells present in the tissue. T cells are known to interact in this way with self-peptides (a phenomenon known as tonic stimulation). Tonic stimulation is important for T cell homeostasis and may render T cells more readily activatable upon foreign antigen exposure (Stefanova et al., 2002). Perhaps the higher, yet statistically insignificant, increase in MHC-I at pre-B2 may have provided more tonic stimulation and heightened the T cell response post-B2 when, perhaps, a damage-specific epitope was displayed on MHC-I.

Interestingly, CD8+ T cells seemed to aggregate around and invade necrotic muscle fibers (necrotic fibers were very rarely observed). A representative image of this phenomenon is shown in Figure 1.4B. This may suggest that T cells promote muscle damage by inducing muscle fiber death, or may serve a phagocytic role following fiber damage. The interpretation that CD8+ T cells play a deleterious roll in muscle by promoting muscle fiber damage is supported by the observation that depletion of these cells significantly reduced pathologic symptoms in murine models of Duchenne muscular dystrophy (Spencer et al., 2001) and dysferlinopathy (Farini et al., 2012). However, given the present observation that CD8+ T cells are only significantly increased after B2 (Figure 1.4C) when evidence of muscle damage was reduced (Figure 1.1A and B), suggests that these cells do not exacerbate exercise-induced damage in nondystrophic human muscle. Rather, the present data are more consistent with a recent rodent study, showing that CD8+ T cells facilitate muscle repair via MCP-1 chemokine expression, thus assisting in the recruitment of tissue-repairing macrophages (Zhang et al., 2014).

Study Limitations

Limitations of the present study include 1) the potential inflammation promoting effect of the biopsy procedure, and 2) the single 48-hour sampling time point following exercise. Though the biopsies were taken 3 days apart, studies have shown that multiple muscle biopsies taken from the same muscle in close proximity are capable of promoting changes in inflammatory cytokine transcript level in subsequent biopsy samples (taken 2 days apart) independent of exercise (Van Thienen et al., 2014). On the contrary, other studies have shown markers of damage and inflammatory events in only the exercised, but not the rested, limbs (Mackey et al., 2008;Lauritzen et al., 2009). To reduce the effects of the previous biopsy procedure on the measured inflammatory markers, the post-B2 biopsy was always taken proximal to the previous biopsy site with the needle angled away from the previous biopsy site. This practice has been shown to minimize inflammation-related artifact from a previous biopsy procedure (Van Thienen et al., 2014). Moreover, we examined cytokine concentrations at the protein level, which appears to be far less affected by the biopsy procedure compared to mRNA expression (Van Thienen et al., 2014). It is also not likely that the biopsy performed prior to B2 negatively impacted LC done on the following day. A previously published paper that used the same subjects presently reported showed that the amount of work done in B1 (42.3 ± 9 kJ) was not statistically different than that done in B2 (43.3 ± 17 kJ) ($p = 0.42$) (Hyldahl et al., 2015).

In order to minimize the total number of biopsies, yet assess inflammation markers during the period of peak muscle soreness and strength loss, we chose to sample 48 hours following each bout of LC. It is possible that changes in some of the inflammatory markers were missed if they occurred earlier or later than 48 hours after exercise. It is possible that the

magnitudes of the inflammatory response at B1 and B2 were not different early after exercise prior to the 48-hour time point, and that inflammation post-B2 simply persisted longer.

Conclusions

The data presented here do not support the hypothesis that blunted inflammation following a repeated bout of LC explains the attenuated symptoms that accompany the repeated bout effect. Rather, we observed increased inflammation in muscle tissue concomitant with reduced evidence of muscle damage following B2. Moreover, the inflammatory environment in the muscle following B2 seemed to be optimized to facilitate recruitment of immune cells as evidenced by increased levels of chemokines MCP-1 and IP-10. Indeed, muscle-infiltrating $CD68^{+}$ macrophages as well as CD8+ T cells were also elevated following B2 and were significantly correlated with the content of both chemokines. Collectively, the data suggests that in response to an initial bout of damaging exercise the muscle becomes more effective at recruiting immune cells following a repeated bout of LCs and that these cells may facilitate accelerated repair to the muscle and thereby contribute to the repeated bout effect. This study also provides the first observation that CD8+ T cells may be involved in the adaptive processes of healthy human skeletal muscle in response to a repeated bout of damaging exercise. Lastly, the increases in CD8+ T cells were seen in the absence of significant increases in MHC-I.

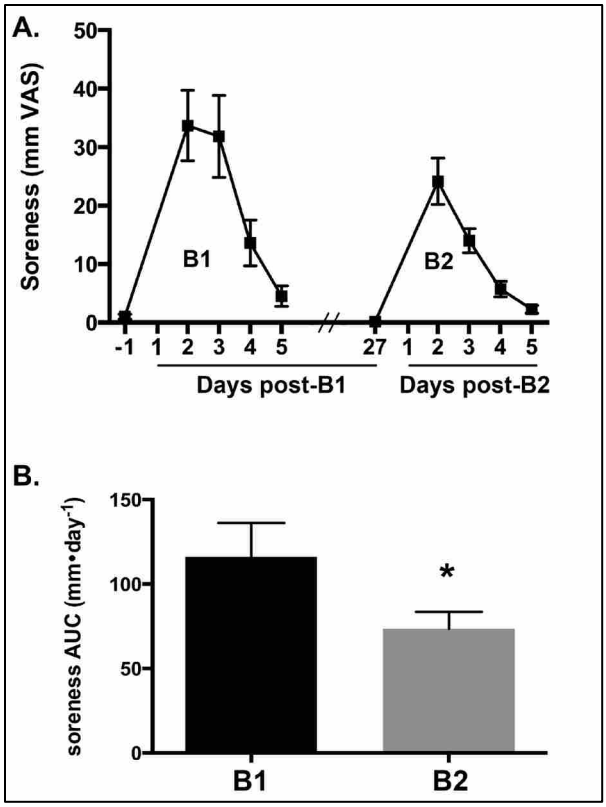

Figure 1.1 Delayed onset muscle soreness as an indirect marker of muscle damage. **(A)** Soreness response curves measured on a visual analog scale (VAS) for 1 day before and 5 days following the first (B1) and second bouts (B2) of lengthening contractions (LC). **(B)** The area under the curve (AUC) for muscle soreness for B1 and B2. * Indicates significant difference (paired t-test, $p < 0.05$).

Table 1 Cytokine concentrations from skeletal muscle biopsy samples 24 hours before (Pre) and 48 hours after (Post) 2 bouts of exercise consisting of 300 maximal lengthening contractions (LC) separated by 27 days. Data are means ± SEM of 10 detectable cytokines. Cytokines were deemed undetectable when the majority (> 50%) of the observations were below the minimum detection limit. The minimum detection limit ranged from 0.82 pg/ml to 2.18 pg/ml. The 19 undetectable cytokines were: epidermal growth factor (EGF), Eotaxin, granulocyte-macrophage colony-stimulating factor (GMCSF), interferon gamma (IFNy), interleukin 10 (IL-10), interleukin 12 p40 (IL-12p40), interleukin 12 p70 (IL-12p70), interleukin 15 (IL-15), interleukin 17 alpha (IL-17A), interleukin 1 receptor antagonist (IL-1 RA), interleukin 1 alpha (IL-1a), interleukin 1 beta (IL-1b), interleukin 2 (IL-2), interleukin 3 (IL-3), interleukin 8 (IL-8), macrophage inflammatory protein 1 alpha (MIP-1a), macrophage inflammatory protein 1 beta (MIP-1b), tumor necrosis factor alpha (TNF-a), tumor necrosis factor beta (TNF-b).
* indicates statistically significant difference from bout 1 pre-LC.

Cytokine	Abbreviation	Bout 1 LC		Bout 2 LC	
		Pre ($pg \cdot ml^{-1}$)	**48 h Post ($pg \cdot ml^{-1}$)**	**Pre ($pg \cdot ml^{-1}$)**	**48 h Post ($pg \cdot ml^{-1}$)**
Interferon gamma-inducible protein 10	IP-10	10.1 ± 1.3	25.6 ± 7.6	13.3 ± 2.4	35.7* ± 12.9
Monocyte chemoattractant protein 1	MCP-1	6.8 ± 0.7	10.3 ± 1.9	11.5 ± 1.14	51.6* ± 29.1
Interleukin 4	IL-4	25.6 ± 4.7	24.7 ± 5.5	18.1* ± 4.5	12.5* ± 2.1
Granulocyte colony-stimulating factor	GCSF	23.8 ± 5.3	32.1 ± 4.5	32.2 ± 4.5	27.2 ± 4.3
Interferon alpha 2	IFNa-2	34.5 ± 3.1	35.3 ± 3.0	33.9 ± 2.6	31.3 ± 2.1
Interleukin 13	IL-13	5.9 ± 0.8	6.6 ± 0.7	5.9 ± 0.5	5.7 ± 0.5
Interleukin 5	IL-5	2.9 ± 0.2	2.8 ± 0.2	2.8 ± 0.2	2.4 ± 0.1
Interleukin 6	IL-6	13.0 ± 1.6	2.8 ± 1.7	13.0 ± 1.6	11.0 ± 1.3
Interleukin 7	IL-7	2.5 ± 0.1	2.4 ± 0.1	2.4 ± 0.2	2.4 ± 0.1
Vascular endothelial growth factor	VEGF	25.8 ± 2.0	31.3 ± 3.6	27.9 ± 2.5	26.3 ± 3.9

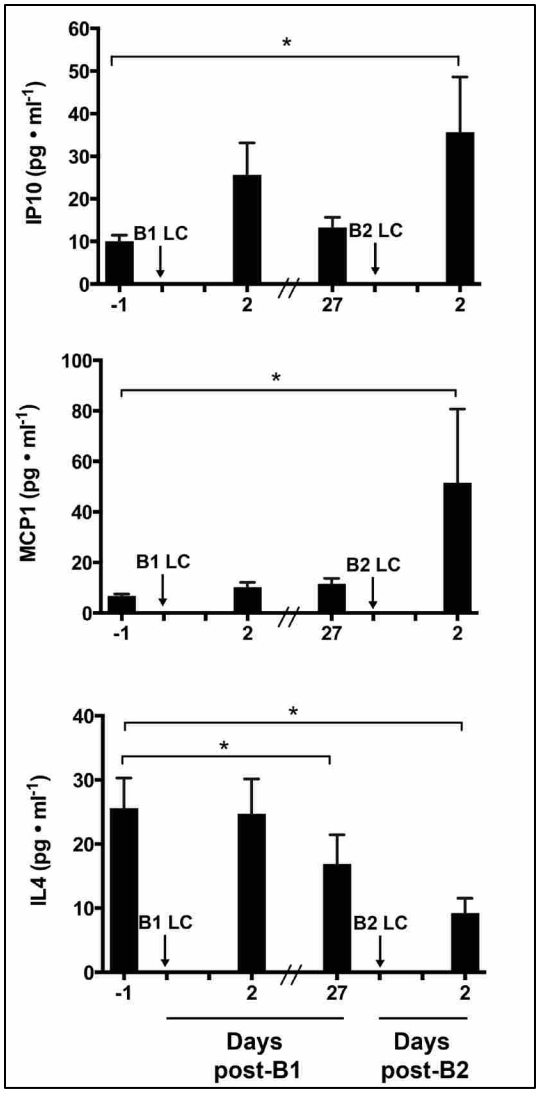

Figure 1.2 Intramuscular cytokine protein concentration before and 2 days following the first (B1) and second bouts (B2) of lengthening contractions (LC). Abbreviations: Interferon gamma-inducible protein 10 (IP-10), Monocyte chemoattractant protein 1 (MCP-1) interleukin 4 (IL-4). * Indicates significant differences between two samples (one-way repeated measures ANOVA with Tukey's HSD, $p < 0.05$).

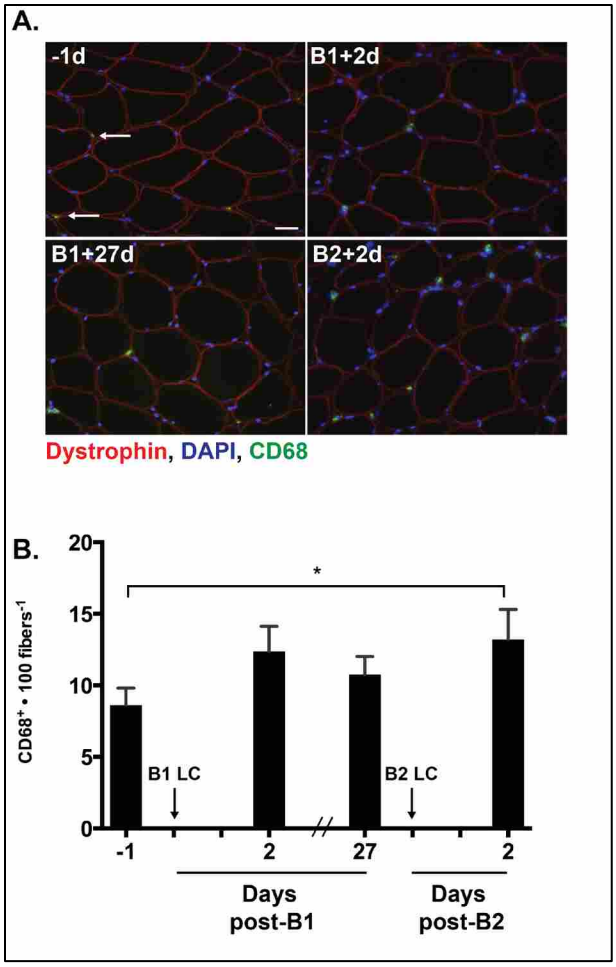

Figure 1.3 Muscle-infiltrating CD68+ macrophages. **(A)** 8 μm thick muscle cross section stained for dystrophin (red), CD68 (green) and DNA (blue). All four images were taken from muscle samples from the same subject 1 day before bout 1 (B1) of damaging lengthening contractions (LC) (−1d), 2 days after B1 (B1 + 2d), 27 days after bout 1 (B1 + 27d) and 2 days after bout 2 (B2) (B2 + 2d). Arrows indicate a CD68+ macrophage as evidenced by CD68 immunoreactivity surrounding a blue nucleus. **(B)** intramuscular $CD68^{+}$ macrophage enumeration per muscle fiber before and 2 days following the first (B1) and second bout (B2) of damaging lengthening contractions (LC). * Indicates significant difference (one-way repeated measures ANOVA with Tukey's HSD, $p < 0.05$). Scale bar = 20 μm.

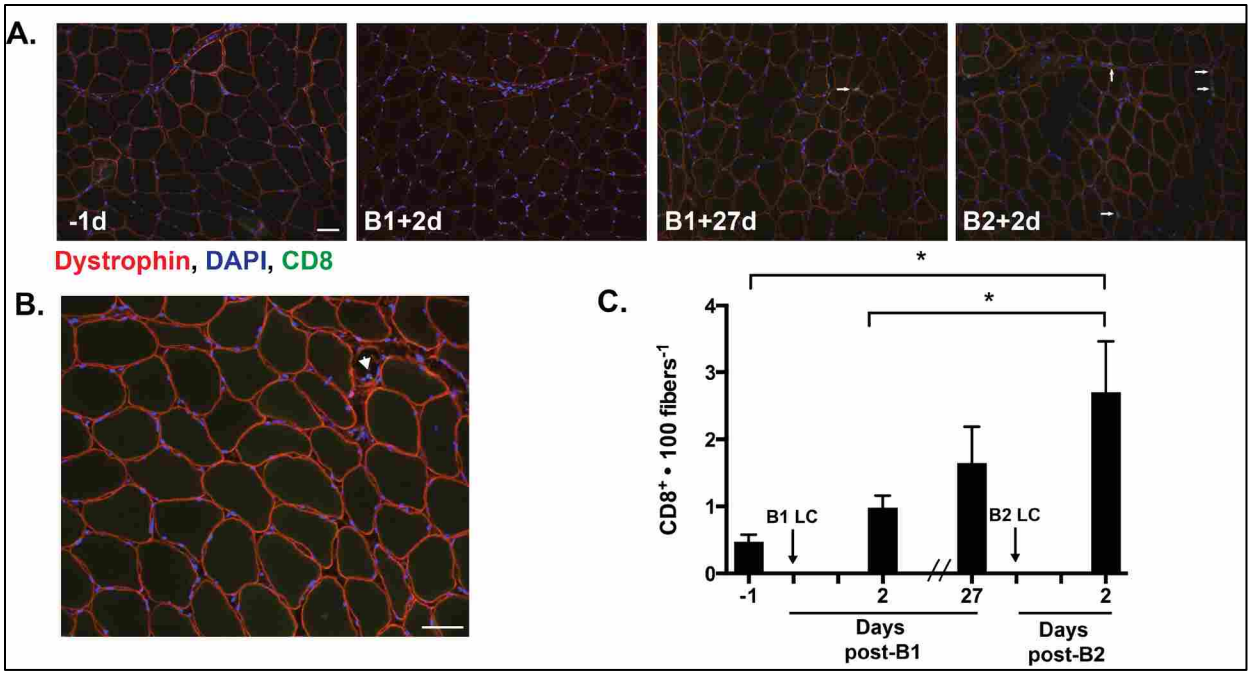

Figure 1.4 Muscle-infiltrating CD8+ T cells. (A) 8μm thick sections stained for dystrophin (red), DNA (blue), and CD8 (green). All four images were taken from muscle samples 1 day before bout 1 (B1) of damaging lengthening contractions (LC) (−1 d), 2 days after B1 (B1 + 2 d), 27 days after bout 1 (B1 + 27 d) and 2 days after bout 2 (B2) (B2 + 2d). The arrows show a CD8+ T cell as evidenced by CD8 immunoreactivity localized around a DAPI-positive nucleus. Merged image reveals the CD8+ T cell invading an apparently necrotic fiber. (B) This merged image shows a subject with an abnormally high number of interstitial CD8+ T cells. (C) CD8+ T cell enumeration per muscle fiber before and 2 days following the first (B1) and second bout (B2) of damaging lengthening contractions (LC). * Indicates significant difference (one-way repeated measures ANOVA with Tukey's HSD, $p < 0.05$). Scale bar = 50 μm.

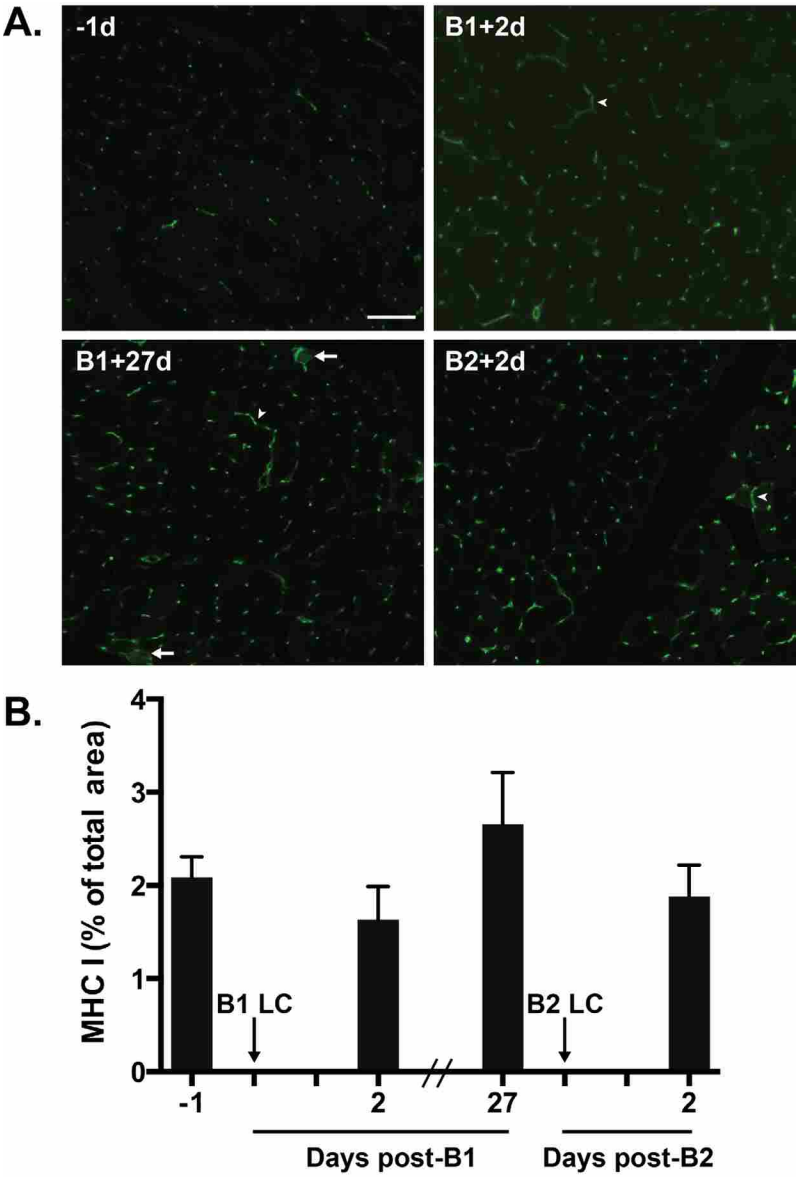

Figure 1.5 Major histocompatibility complex class 1 (MHC-I) in skeletal muscle tissue. **(A)** A representative florescent image of 8 μm thick muscle cross sections stained for MHC-I. All four images were taken from muscle samples from the same subject 1 day before bout 1 (B1) of damaging lengthening contractions (LC) (−1 d), 2 days after B1 (B1 + 2 d), 27 days after bout 1 (B1 + 27 d) and 2 days after bout 2 (B2) (B2 + 2 d). Arrowheads show MHC-I positive sarcolemmal immunoreactivity. Arrows show MHC-I positive sarcoplasmic staining. **(B)** Percent of total muscle section positive for MHC-I before and after B1 and B2 of LC. No significant differences observed (one-way repeated measures ANOVA with Tukey's HSD, $p < 0.05$). Scale bar = 100 μm.

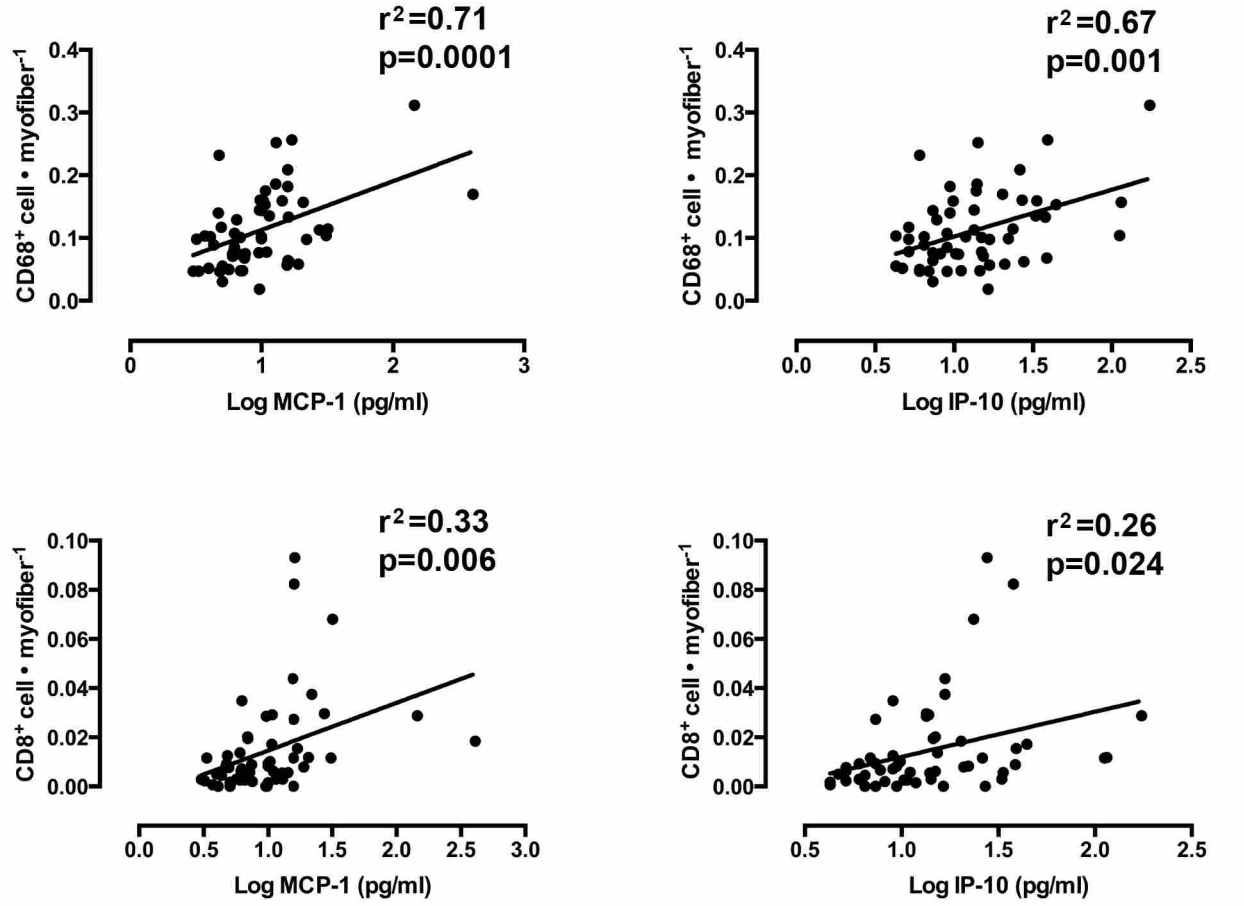

Figure 1.6 The association between the number of muscle-infiltrating CD68+ macrophages and CD8+ T cells and the protein content of monocyte chemoattractant protein 1 (MCP-1) and interferon gamma-inducible protein 10 (IP-10). (Mixed models linear regression, $\alpha = 0.05$).

Study II A Time Course Characterization of Muscle T Cell Accumulation Following Damaging Muscle Contractions

Abstract

CD8+ T-cells are known to infiltrate human skeletal muscle in severe muscle diseases including Duchenne muscular dystrophy and inflammatory myopathies. Recent evidence suggests that CD8+ T cells may facilitate repair in damaged nonmyopathic skeletal muscle. Investigations into CD8+ T cells following contraction-induced damage is quite limited. The general time course of CD8+ T cell accumulation following damaging contractions, and whether there is a sexually dimorphic response, is not known. In this investigation, six men and three women volunteers (22.2 ± 2.4 years of age) did 300 (30 sets of 10 reps) maximal-effort lengthening contractions (LC) of the knee extensor muscles using an isokinetic dynamometer. Before LC, a muscle biopsy was taken from the vastus lateralis of the nonexercised leg. After LC, three more biopsies were taken from the exercised leg at 3, 24, and 72 hours post-LC. Compared to pre-LC (207.2 ± 37 Nm), maximal isometric strength was significantly reduced immediately after (106 ± 45.7 Nm), 24 hours after (107 ± 50 Nm), and at 72 hours after LC (112 ± 70 Nm) ($p < 0.05$). Strength loss between men and women was not different ($p > .05$) indicating that the degree of damage was similar between genders. Muscle-infiltrating CD8+ T cells were enumerated via immunohistochemistry and fluorescence microscopy. A linear mixed models analysis revealed a significant increase in CD8+ cells at 72 hours post-LC relative to baseline (5 fold, $p = 0.0008$), 24 hours post-LC (3.2 fold, $p = 0.0016$), and 3 hours post-LC (3.1 fold, $p = 0.018$). Moreover, a significant Sex X Time interaction ($p = 0.01$) indicated that females had a greater CD8+ T cell response to damaging exercise than males. In conclusion, these data show that CD8+ T cells infiltrate healthy, nonmyopathic human muscle in response to exertional

damage, and that infiltration peaks at or later than 72 hours after damage. Moreover, these data suggest that this response is greater in women.

Introduction

Various cellular members of the immune system including mast cells, neutrophils, monocytes/macrophages, eosinophils, and T cells are involved in the inflammatory response following muscle damage (Cote et al., 2008;Tidball, 2017). The relationship between muscle and the immune system during the process of repair and regeneration following injury is complex. On one hand, each of these cells has been shown to carry out necessary, if not crucial, functions in the repair/regeneration process. On the other hand, each of these cells are also capable of hindering the repair/regeneration process by either exacerbating muscle damage or promoting a maladaptive repair response such as fibrosis. Whether these inflammatory cells help or hinder the muscle repair process depends on several factors such as the nature/severity of the injury, or the underlying pathology/demographic of the organism (Tidball, 2017).

Much of the available literature on the interaction between the immune system and muscle in the context of muscle repair/regeneration has been done in animals using models of traumatic muscle injury. A smaller body of literature shows that immune cells are also involved in muscle repair following exercise-induced (contraction-induced) muscle damage, which is usually less severe than typical models of traumatic injury such as cryo or myotoxic injury. Neutrophils and macrophages are the most well-known immune cell types to infiltrate muscle following contraction-induced damage (MacIntyre et al., 2000;Stupka et al., 2000;Stupka et al., 2001). Much less is known about the participation of other immune cells in the repair process following contraction-induced damage (Peake et al., 2017).

A few recent investigations have found that CD8+ T cells accumulate in human muscle following contraction-induced muscle damage (Marklund et al., 2013;Deyhle et al., 2015). Both of these studies only measured CD8+ T cells at a single time point after contraction-induced damage (48 or 28 hours). Thus the time course of muscle T cell accumulation following damaging contractions is not yet known.

Previous studies have shown that immune cell accumulation in muscle is different between men and women (MacIntyre et al., 2000;Stupka et al., 2000;Stupka et al., 2001). Therefore, another outstanding question is whether muscle T cell accumulation following muscle damage is similar between men and women. Answering these descriptive questions would provide prerequisite information for more in-depth future studies such as the role T cells play in muscle repair or adaptation from contraction-induced damage.

The purpose of this study was to provide a more detailed time course of muscle T cell flux following contraction-induced damage in humans and investigate whether there muscle T cell flux is different between men and women.

Methods

Subjects

Six men and three women (22.2 ± 2.4 years of age) volunteered to participate in this study. The subjects were informed of the study procedures and potential risks of their participation prior to signing an informed consent document. All subjects were moderately active but were not participating in any regularly scheduled physical activity or strength training within the past 6 months. All subjects agreed to refrain from starting any new physical activity and taking any oral or topical analgesics for the duration of the study. The BYU institutional review board approved all study procedures prior to beginning data collection.

Study Design

The subjects visited the lab 4 times. During the first visit, unilateral knee extensor strength of a randomly selected (exercised) leg was assessed on a dynamometer (Biodex Medical Systems, Shirley, NY). After the strength assessment, a baseline muscle biopsy was taken from the vastus lateralis of the opposite (control) leg. The following day, subjects returned to the laboratory to complete a bout of lengthening contractions (LC) to cause damage to the knee extensors (Deyhle et al., 2015;Deyhle et al., 2016). Strength was assessed again within 5 minutes of completing the LC. The subjects then rested in the laboratory for 3 hours. After the rest period, a muscle biopsy was obtained from the vastus lateralis of the exercised limb. A follow up strength assessment and biopsy sample from the exercised leg was obtained at 24 and 72 hours after the LC.

Strength Assessment and Lengthening Contractions

The dynamometer (Biodex Medical Systems, Shirley, NY) position settings were adjusted to fit each individual such that the rotational axis of the knee aligned with that of the dynamometer when the individual was securely seated in the chair of the dynamometer. For the strength assessments, subjects did maximal concentric isokinetic contractions of the knee extensor muscles (60^{o} per second). The investigator provided verbal encouragement for the subject to exert maximal effort during the isokinetic strength test. The peak torque measured among 3 contractions was recorded. For the bout of muscle damaging contractions, the subject completed 300 maximal-effort LCs (30 sets of 10 reps with 1 minute of rest between sets). For each contraction, the subject resisted the movement of the lever arm, as it rotated the knee from 30^{o} to the individual's maximal knee flexion (about 110^{o} flexed) at a rate of 60^{o} per second.

Muscle Biopsy

Muscle biopsy samples were obtained from the vastus lateralis as described previously (Deyhle et al., 2015). Briefly, the skin was disinfected and 2% lidocaine was injected as a local anesthetic. Next, a small incision was made through the skin and fascia over the middle of the vastus lateralus. A Bergstörm biopsy needle was inserted through the incision and the sample was extracted with a suction-assisted method. Percutaneous muscle biopsies were then collected through a manual suction method. The first biopsy incision was made approximately 15 cm proximal to the insertion of the vastus lateralis of the nonexercised leg. Efforts were made to minimize inflammatory artifact of previous biopsies by taking subsequent biopsies (3rd and 4th) more proximal than prior biopsies (Van Thienen et al., 2014). Muscle samples were preserved for immunohistochemistry by freezing in liquid nitrogen-cooled isopentane and were stored at −80ºC.

Immunohistochemistry

Muscle samples were sectioned using a cryostat (8 µm thick) and mounted on glass slides. After drying for 10 minutes, samples were fixed in PBS with 2% paraformaldehyde for 10 minutes at room temperature. Samples were then washed and stained for CD8 as previously described (Deyhle et al., 2015). Briefly, samples were incubated in primary antibodies overnight at 4°C (CD8 diluted 1:50 in PBS and dystrophin 1:500). The next day, samples were washed and incubated in secondary antibodies diluted 1:100 in PBS for 30 minutes at 37°C. DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride) was added to the secondary antibody solution to stain nuclei. Primary antibodies used were CD8, mouse monoclonal IS623 (Dako, Glostrup, Denmark) and dystrophin, rabbit polyclonal (ab15277; Abcam, Cambridge, United Kingdom).

Quantification of Muscle T Cell Content

The entire muscle cross section stained for CD8+ T cells was imaged using Olympus IX73 fluorescence-capable inverted microscope using the 20X objective. Ten to twenty nonoverlapping images were captured and analyzed using Olympus cellSens Software (Tokyo, Japan). CD8 positive T cells were identified by green (Alexa Fluor^{TM} 488) immunoreactivity around a blue (DAPI+ nucleus). The total imaged area was quantified, and the number of CD8 positive T cells was expressed per square millimeter of imaged area.

Statistical Analysis

A linear mixed model was used to analyze these data. Time (pre, post, 24-hour post, and 72-hour post, Sex (male or female) and the interaction between Time and Sex were included as fixed effects and a subject identifier variable was included as a random effect and was crossed with Time and nested with Sex. A Tukey's HSD test was used to correct for multiple pairwise comparisons. Differences were considered statistically significant when $p < 0.05$. Normality of the data was determined by examining the residual by predicted plot, and transformations were applied when appropriate. Data are presented as mean ± SD.

Results

Muscle Strength

The loss and restoration of muscle strength is a reliable and valid indicator of muscle damage and repair (Paulsen et al., 2012). The muscle strength data are shown in Figure 2.1. Overall, men were stronger than women, but this did not reach statistical significance (Sex, $p = 0.08$), probably due to the low sample size in the female group. A significant loss in muscle strength was observed after LC that remained out to 72 hours post-LC (Time, $p = 0.0001$). Men and women lost strength similarly over time (Sex X Time, $p = 0.9$)

Muscle T Cell Content

Muscle CD8 T cell data were analyzed on the log scale and are displayed in Figure 2.2. Compared to pre-LC values (0.5 cells/mm^2), the number of intramuscular CD8 positive cells did not change 3 hours post-LC (1.45 ± 1.25 cells/mm^2) or 24 hours post-LC (1.35 ± 1.7 cells/mm^2). However, significantly more T cells were present 72 hours post-LC (5.5 ± 7.15 cells/mm^2, p = 0.0008). Moreover, there was a significant Sex by Time interaction (p = 0.0096), whereby women exhibited a greater increase in intramuscular CD8 positive cells 72 hours post-LC than men (12.2 ± 32.9 vs. 2.2 ± 2.4 cells/mm^2, p = 0.01).

Discussion

This study confirms previous reports (Marklund et al., 2013;Deyhle et al., 2015) that CD8 positive T cells accumulate in human muscle following damaging muscle contractions. The primary purpose of this study was to provide a more detailed picture of the flux of muscle CD8 T cell content following contraction-induced damage. Thus far, studies have only observed T cells following damaging exercise at a single time point. Deyhle et al. (Deyhle et al., 2015) observed a significant increase in intramuscular T cells at 48 hours following contraction-induced damage. Marklund et al. (Marklund et al., 2013) measured intramuscular T cells 28 hours after prolonged exercise. In the present investigation, we measured muscle T cells at 3 hours, 24 hours, and 72 hours in order to get a broader picture of the CD8 T cell accumulation in muscle following damaging muscle contractions. These results show that T cells did not significantly increase over preexercise levels until 72 hours after LC. Thus, it appears that the peak of accumulation following contraction-induced damage occurs at or later than 72 hours following lengthening contractions. This finding is similar to previous studies on traumatic muscle injury (Zhang et al., 2014;Fu et al., 2015), which showed that CD8 T cell accumulation peaked between 3 and 5 days

after cardiotoxin exposure. Thus, it appears that muscle T cell accumulation after contraction-induced damage follows a similar time course to accumulation following traumatic muscle injuries.

Few studies have examined sex differences in the muscle inflammatory response following damaging contractions. Similar to the present finding, that women had a greater increase in muscle T cells at 72 hours than men, two previous investigations found that women had greater muscle neutrophil accumulation following damaging contractions than men (MacIntyre et al., 2000;Stupka et al., 2001). In contrast, another investigation found that men tended to have a greater increase in muscle white blood cells in general than women ($p = 0.052$). The reason why women may display a greater increase in immune cell subsets in muscle following damage is not known. Importantly, there was no difference in muscle damage between men and women in this study (as measured by strength loss, Figure 2.1).

Women and men usually have a similar muscle damage responses to a given damaging stimulus (Hubal and Clarkson, 2009). The present investigation affirms this given that there was no effect of the Sex X Time interaction for muscle force loss ($p = 0.9$), meaning that the degree of muscle damage was similar between men and women. Therefore, the difference in CD8 T cell accumulation cannot be attributed to differences in muscle damage. Interestingly, women are generally more likely than men to suffer from autoimmune disorders (Ngo et al., 2014). Thus, it is possible that women are more prone to react to self-peptides (self-antigens), and that the heightened inflammatory response to muscle damage is a manifestation of that tendency.

The role of these T cells in the context of contraction-induced damage and whether they are beneficial or detrimental to the repair process cannot be determined from the present data. It may be that they are carrying out important regulatory roles on the infiltration of other immune

cells, as they have been shown to do in animal models of severe muscle injury (Zhang et al., 2014). More studies are needed to elucidate the functional role(s) of these cells in the repair process following contraction-induced injury. Studies using knockout animals lacking T cells could be useful to answer these questions.

In conclusion, this study found the peak of CD8 T cell accumulation in muscle following contraction-induced damage is at or later than 72 hours post-LC, and that this response is greater in women than in men. By providing a more detailed time course of muscle CD8 T cell accumulation, these results could be useful for future investigations into the role of T cells in muscle repair and adaptation from contraction-induced damage. These results also could encourage investigation into the mechanisms and consequences of a sexually dimorphic inflammatory response to damaging muscle contractions.

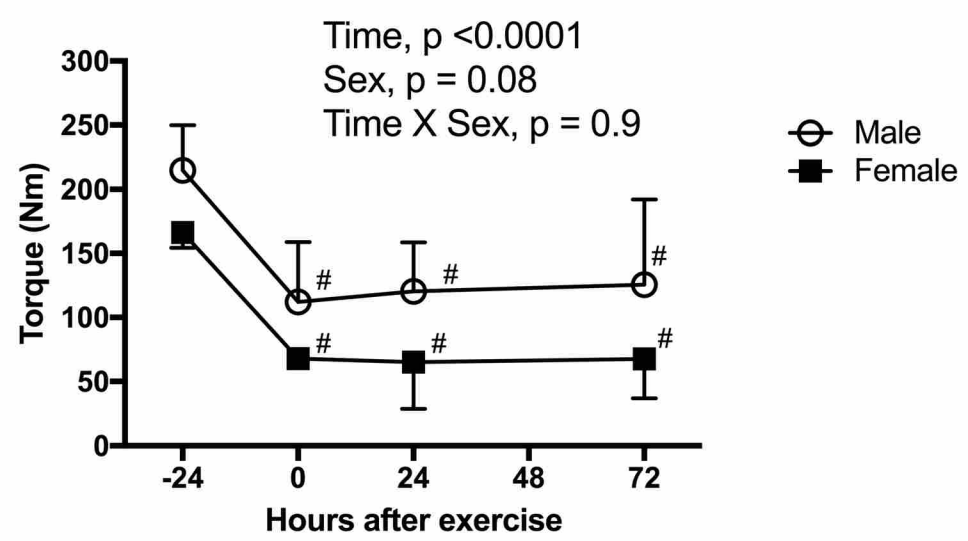

Figure 2.1 Isokinetic knee extensor strength loss following a bout of lengthening contractions. This figure shows peak isokinetic (60°/sec) strength in Nm (mean ± SD) of the knee extensor muscles 24 hours before (−24) and 0, 24 and 72 hours after 300 maximal effort lengthening contractions (30 sets of 10 reps) of the knee extensors in 6 men (O) and 3 women (■). Men and women lost strength compared to baseline values ($p < 0.05$, #). Strength loss between men and women was similar (Sex X Time, $p = 0.9$). Men tended to be stronger than women (Sex, $p = .08$).

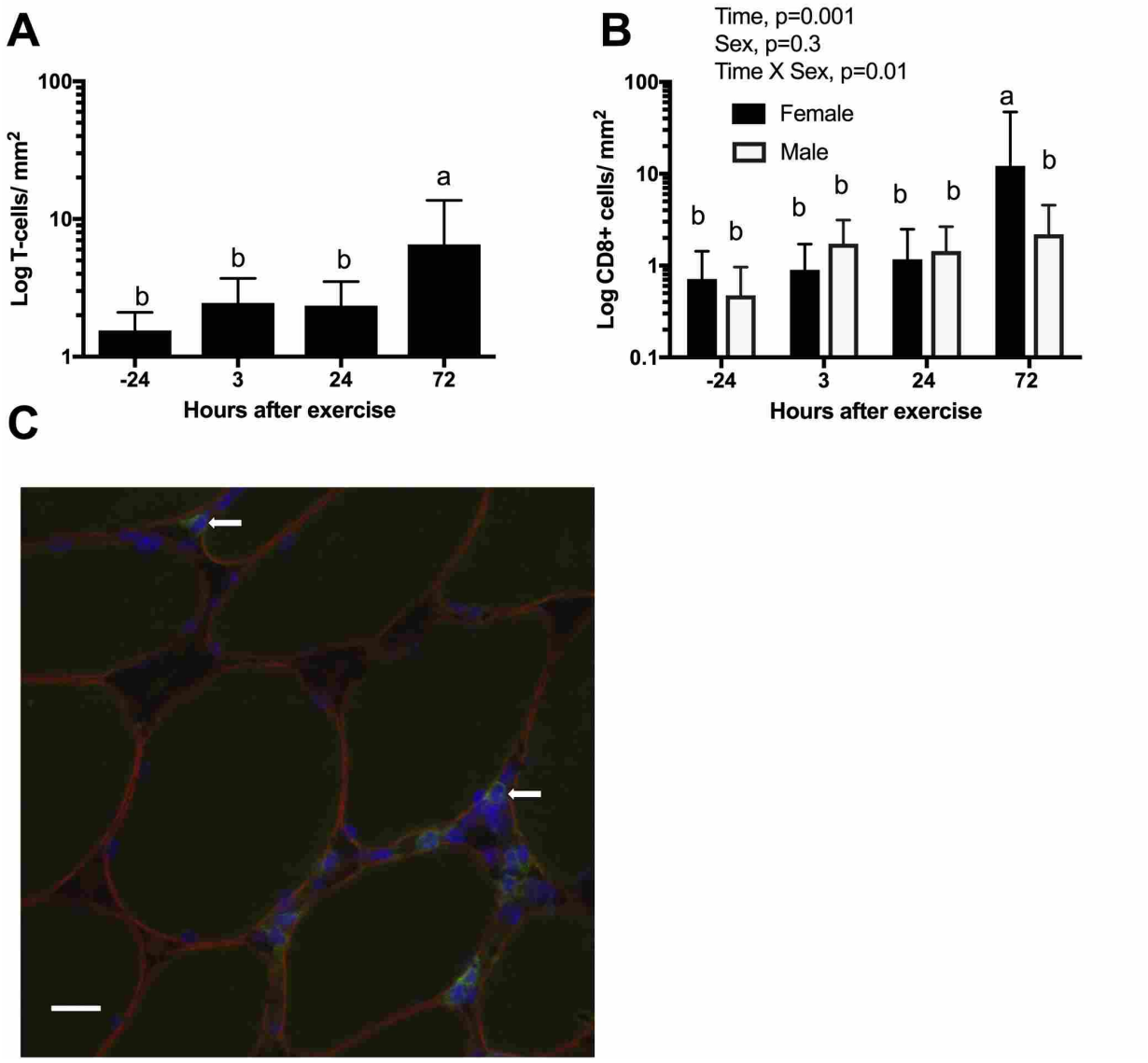

Figure 2.2 CD8+ cells per mm^2 of imaged area increased significantly 72 hours after damaging exercise. **(A)** This figure shows the log of T cells per mm^2 of imaged muscle (mean ± SD) one day before (-24), 3, 24 and 72 hours after 300 maximal effort lengthening contractions (30 sets of 10 reps) of the knee extensors. **(B)** This figure shows the same data separated by sex. Significantly more CD8+ cells were present in the muscle 72 hours after exercise (*, p = <0.05). Women showed a greater response than men (Time X Sex, p = 0.01). b = significantly different than a, p < 0.05). **(C)** A representative micrograph from the muscle sample of a high-responding subject 72 hours after lengthening contractions. Arrows point out CD8+ cells. CD8+ cells were identified by CD8 staining around a DAPI+ nucleus. Green = CD8, Blue = DNA, Red = Dystrophin. Scale bar = 20 µm.

Study III The Role of T Cells in the Repeated Bout Effect

Abstract

Skeletal muscle rapidly adapts to contraction-induced damage such that it is subsequently resistant to damage. This phenomenon is often called the repeated bout effect (RBE). Interestingly, following damaging contraction on one muscle group, the contralateral analogous muscle group also acquires resistance to damage. This is known as the contralateral RBE (cRBE). Studies show immune cells and inflammation are involved in bringing about the RBE and cRBE. This study was done to 1: establish a model of the RBE and cRBE in rats following lengthening contractions (LC), 2: characterize T cells in the muscle inflammatory response to single and repeated bouts of LC using flow cytometry, and 3: to test the hypothesis that T cells formerly exposed to muscle damage could protect muscle against damage caused by LC. Animals that did two bouts of LC separated by 2 weeks sustained less damage after the second bout. However, rats that did a repeated bout of LC on the opposite limb showed no resistance to damage. CD4+, CD8+ and regulatory T cells increased in muscles that sustained damage. In rats that were protected from damage (RBE), a minimal increase in T cells was observed. Adoptive transfer of T cells from rats that had previously done muscle-damaging exercise did not confer muscle damage protection to recipient rats following LC. In conclusion, the RBE, but not the cRBE was observed in rats, and T cells infiltrated muscle damaged by LC, but they do not appear to contribute to the RBE in the same way that they contribute to adaptive immunity.

Introduction

Skeletal muscle is susceptible to damage from contractions of exercise. Lengthening contractions (LC) are especially suited to cause muscle damage, particularly when they are done with high force, high strain, and a large number of repetitions (Hyldahl et al., 2014;Hyldahl et

al., 2017). Following a single exposure to contraction-induced damage, protective adaptations are enacted such that the muscle is subsequently resistant to damage from a future stimulus (Clarkson and Tremblay, 1988;Hyldahl et al., 2017). This damage-protective adaptation is commonly called the "repeated bout effect" (RBE).

Interestingly, several studies have found that not only are the damage-experienced muscles resistant to damage, but the protective adaptation is also expressed in muscles that were not previously damaged. Unilateral LC can confer protection to the damage-naïve contralateral, analogous muscle group (Howatson and van Someren, 2007;Chen et al., 2016). This special case of RBE has been termed the contralateral RBE (cRBE). Importantly, the cRBE is less potent than the RBE. That is, the magnitude of protection observed on the contralateral, damage-naïve muscle group is typically about 50% of the protection observed on the damage-experience muscle group (Hyldahl et al., 2017). The cRBE is also less persistent, lasting about 4 weeks (Chen et al., 2016) compared to the RBE, which remains up to 6 to 9 months (Nosaka et al., 2001)

Several data-supported mechanisms of the RBE have been proposed. These include changes in neural recruitment (McHugh, 2003;Howatson et al., 2011), muscle extracellular matrix (ECM)-related changes (Boppart et al., 2008;Mackey et al., 2011;Hyldahl et al., 2015), increased tendon compliance (Lau et al., 2015), and altered immune system (inflammatory) responses (Lapointe et al., 2002a;Hubal et al., 2008;Deyhle et al., 2015). However, a complete and definitive understanding of the mechanism(s) behind the RBE and cRBE remains to be established.

Given that immune cells play an integral role in the muscle damage and repair/ regeneration process (Tidball and Villalta, 2010;Tidball, 2017), an immune system-related

explanation of the RBE is a reasonable candidate. Several immune cell types including neutrophils (Nguyen et al., 2005;Pizza et al., 2005) macrophages (Wehling et al., 2001;Lapointe et al., 2002b), eosinophils (Wehling-Henricks et al., 2008) and T cells (Spencer et al., 1997;Kohno et al., 2011) can exacerbate muscle damage following injury. Yet, each of these immune cell types is also known to carry out beneficial and often crucial functions in effective muscle repair/regeneration (Teixeira et al., 2003;Tidball and Wehling-Henricks, 2007;Heredia et al., 2013;Zhang et al., 2014). Therefore, an immune system-related adaptation to damaging contractions, whereby any damage-worsening effects of the immune cells are minimized and beneficial muscle repairing effects are maximized, could contribute to the RBE. One potential immune system-related mechanism of the RBE is that inflammation is reduced following a repeated bout, which leads to less "secondary damage" caused by the immune cells (Pizza et al., 1996;Lapointe et al., 2002b). On the other hand, more recent data show that immune cells might be playing an active role in the acquisition of the RBE, as blunting neutrophil infiltration hinders the acquisition of the RBE (Lockhart and Brooks, 2008). Moreover, some studies have shown that inflammation is not reduced, but perhaps even increased, following a repeated bout of damaging contractions (Stupka et al., 2001;Hubal et al., 2008;Deyhle et al., 2015). For example, we recently found that CD8 T cells (together with other evidence of inflammation) accumulated in human muscle to a significantly greater degree after a repeated bout of lengthening contractions compared to after the initial bout (Deyhle et al., 2015).

T cells are a heterogeneous class of immune cells with various subsets. These distinct subsets are identified by characteristic expression of cell surface receptors including CD4 or CD8, as well as transcription factors including Foxp3, GATA3, or T-bet. In recent years, it has become clear that T cells are quite important for successful muscle repair and regeneration

following traumatic muscle injury (Burzyn et al., 2013;Zhang et al., 2014;Fu et al., 2015;Kuswanto et al., 2016). Whether T cells play a role in repair from, and/or adaptation to contraction-induced damage remains unclear. The population of muscle infiltrating T cells after contraction-induced damage has not been well characterized. To date, studies have only measured CD8 positive T cells using immunohistochemistry following contraction-induced damage (Marklund et al., 2013;Deyhle et al., 2015). Therefore, it is not clear what other T cell subsets might be responsive to contraction-induced damage.

Classically, T cells are vital members of the immune system. One defining attribute of T cells in the context of immunology is their ability to generate memory. T cells can be educated in response to a primary immune response such that they respond more quickly and effectively to a secondary immune response to the same pathogen. Some principles of T cell adaptive immune function might be carried over to their activity in the context of muscle repair/regeneration. T cells harvested from regenerating mouse muscle were found to express an identical αβ T cell receptor (TCR) sequence arrangement (Burzyn et al., 2013). This same TCR was found repeatedly among different animals in separate experiments. This indicates that muscle damage might generate a damage-indicative peptide with an immunodominant epitope that T cells react to. If T cells react to muscle damage using their TCR, (as they do with their classical immune function), they might generate memory of muscle damage in order to respond more effectively to a subsequent muscle injury. This hypothesis is consistent with our recent observation that CD8 T cells were more plentiful in adapted muscle following a repeated bout of damaging contractions compared to after the initial bout when more damage was sustained (Deyhle et al., 2015). Additionally, because immune cells possess the ability to migrate among tissues, this memory T cell-mediated hypothesis could be a contributing explanation to both the RBE and the cRBE.

The first purpose (study 1) of this investigation was to characterize the muscle T cell infiltrate using multi-color flow cytometry in rats after a single bout of LCs, and after repeated bouts done on either the ipsilateral (same) or contralateral (opposite) limb. We hypothesized that T cell accumulation would be greater following a repeated bout of LC compared to a single bout and that there would be more T cells expressing a marker of memory. In Study 2 of this investigation we tested the hypothesis that T cells are instrumental in the RBE by developing memory of muscle damage on the initial exposure such that they would respond more effectively to a second exposure to support more effective/rapid repair.

Methods

Animals

Male Lewis rats (12 to 14 weeks old, 300 ± 24g) were used for this research. Two to three rats per cage were housed in a room at 22ºC with a 12-hour light and dark cycles. Food and water was available *ad libitum*. Upon arrival at the housing facility, the rats were left unperturbed for 5 to 7 days prior to any testing or experimental intervention. The BYU IACUC approved all animal procedures *a priori*.

Study 1 Design

This study was done to assess the presence of the RBE and the cRBE in rats and to characterize the muscle T cell infiltrate by flow cytometry. To do this, rats were allocated into one of four groups: SB3D, SB14D, RB, cRB. Rats in the SB3D (single bout 3 day, n = 6) group were subjected to a single bout of muscle-damaging LC. An in vivo muscle torque measurement (see details in *Muscle Torque Assessments and Lengthening Contractions)* was done immediately before the bout of LC 5 minutes post-LC, and 3 days post-LC. After the 3-day post-muscle torque assessment, tissue was collected for flow cytometry and histological damage

assessment (see details in *Histology*). The SB14D group (n = 3) underwent the same procedures as SB3D, except the rats survived until 14 days after LC, at which time muscle torque characteristics were assessed and tissue was collected. The RB (repeated bout n = 6) group underwent two bouts of LCs separated by 14 days. Both bouts of LCs were done on the left limb. Muscle torque was assessed immediately before, 5 minutes after, and 3 days after each bout of LC. Tissue was collected after the 3 days after bout 2 muscle torque assessment. The cRB (contralateral repeated bout n = 6) group underwent the same protocol as the RB group (RB) except the second bout of LC, and the associated muscle function tests, were done on the contralateral limb. To account for any effect of limb side, the order of testing was alternated for every rat in the cRB group. For example, the first rat in the cRB group did bout 1 on the left limb and bout 2 on the right limb; the second rat was tested in the opposite order. A group of cage control rats (CC, n = 3) was also used. These rats underwent no muscle contraction protocol, but were used as an unexercised control for the flow cytometry experiment. Figure 3.1A shows a schematic of this study design.

Study 2 Design

The purpose of Study 2 was to test the hypothesis that T cells previously exposed to muscle damage would confer protection against damage from a future stimulus. To test this hypothesis, an adoptive T cell transfer experiment was carried out. T cells were harvested from two donor rats. The damage-naïve (DN) donor rat underwent no muscle contraction procedure. The damage-experienced (DE) donor rat underwent a bout of damaging LC two weeks prior to harvesting T cells. Two groups of recipient rats (n = 5 per group) underwent a bout of LC with a muscle function test before and 5 minutes post-LC. Immediately after the 5-minute post-LC assessment, 2 x 10^6 T cells were injected into the tail vein. The group of naïve adoptive transfer

(nAT) recipient rats received T cells from the DN donor rat, while the experienced adoptive transfer (eAT) group received T cells from the DE donor rat. Muscle damage was then assessed by muscle torque characteristics and histology 3 days after the LC and adoptive cell transfer. Figure 3.1B shows a schematic of this study design.

Muscle Torque Assessments and Lengthening Contractions

Muscle contraction physiology procedures were done in vivo using Aurora Scientific 305c Dual Mode lever system and a Grass S88X stimulator. The rat was anesthetized (2% to 2.5% isoflurane in pure oxygen) and placed supine on a rat in situ apparatus (Aurora Scientific model 806D). The limb was shaved with clippers, cleaned with alternating changes of Providone-iodine and 70% ethanol. Sterile ophthalmic cream (Puralube® Vet Ointment) was applied to the eyes to prevent them from drying. The limb was placed in the rat knee clamp and secured by inserting a 25-gauge needle through the aperture in the knee clamp and into the medial aspect of the femur just proximal to the knee. The paw was then secured to the pedal of the lever system using Transpore^{tm} surgical tape. Twenty-seven gauge subcutaneous needle electrodes (Chalgren Enterprise, Gilroy, CA) were inserted from the lateral side orthogonal to the tibia and just distal to the fibular head around the fibular nerve. Electrode placement was adjusted to optimize isolated activation of the anterior crural muscles during a train of about 10 to 20 tetanic contractions. The minimum voltage needed to elicit peak torque was used. To assess contractile characteristics, a torque frequency test and a torque length test was completed. For the torque frequency test, the nerve was stimulated for 300 ms using 1 ms square waves at 10, 20, 30, 40, 50, 80, 100, 120, 140, and 160 Hz with the ankle joint at 90º. For the peak torque length test, a fused tetanic contraction was achieved by stimulating the nerve for 300 ms using 1 ms square waves at 140 Hz at three different ankle joint angles (97º, 104º and 110º dorsiflexed). To

cause muscle damage, the rats were subjected to a bout of 150 LC as has been done previously (Dipasquale et al., 2011). To do the LCs, an isometric contraction was elicited (1 ms pulses at 140 Hz) with the ankle dorsiflexed approximately 20º from neutral for 200 ms. During the remaining 100 ms of the contraction the ankle joint was plantar flexed through a 40º arch at a rate of 900º/sec. All torque data were normalized to the body mass (kg) for analysis.

Tissue Collection

The rats were euthanized after the final muscle function assessment and while deeply anesthetized. Euthanasia was accomplished by exsanguination (interior vena cava blood draw) followed by a bilateral thoracotomy. Next, the spleen was excised and placed in ice-cold flow cytometry buffer (PBS, 0.5% BSA). Both tibialis anterior muscles were then harvested and partitioned by cross section into thirds. The distal third was weighed and prepared for flow cytometry (see details in *Flow Cytometry*). The middle third was frozen in melting isopentane for histology. The proximal third was flash frozen in liquid nitrogen for future use. All frozen muscle specimens were stored at -80ºC.

Flow Cytometry

To obtain a single cell suspension from muscle samples, approximately 100 mg was minced using razor blades and incubated for 60 minutes (5% CO_2, 37ºC) in an enzyme solution (2 mg/ml of type 2 collagenase, 0.15 mg/ml of DNase I in DPBS with calcium and magnesium). Digesting enzymes were purchased from Worthington Biochemical (Lakewood, NJ). The samples were strained through a 40 µm cell strainer. After straining, samples were centrifuged at 300 x g for 10 minutes at 4ºC and resuspended in 4 ml of 40% Percoll Plus density gradient media (GE Healthcare). The sample was then gently layered over 4 ml of 80% Percoll Plus and centrifuged at 1500 x g, room temperature for 30 minutes with no brake. The cell-containing

interphase was retrieved. To obtain a single-cell suspension from the spleen, the samples were crushed between two frosted microscope slides then filtered (40 μm). Approximately 1 to 5 x 10^5 cells (the whole yield from muscle or an aliquot from spleen sample) were Fc blocked for 10 minutes at 4°C with a CD32 antibody (BD Biosciences, clone D34-485, 1:100). For cell surface staining, the samples incubated for 40 minutes at 4°C with the following fluorochrome-conjugated antibodies, PE-Cy7: CD45 (Biolegend, Clone OX-1, 1:80), PE: CD3 (eBioscience, clone G4.18, 1:80), FITC: CD4 (ThermoFisher, clone W3/25, 1:80), APC: CD8α (eBioscience, clone OX8, 1:80) APC-Cy7: CD127 (ThermoFisher, Polyclonal, 1:12.5). The CD127 antibody was conjugated with the APC-Cy7 Conjugation Kit using the provided protocol (Abcam). Intracellular staining of Foxp3 was accomplished using the Foxp3 Transcription Factor Staining Kit (eBioscience) according to the manufacturer's instructions. Briefly, samples were fixed and permeabilized for 45 minutes at room temperature then incubated for 1 hour at 4°C with PE-Cy5.5: Foxp3 (eBioscience, clone FJK-16s, 1:20). The samples were then analyzed by flow cytometry (Attune Acoustic Focusing Cytometer (Applied Biosystems) or Cytoflex S (Beckman Coulter, Indianapolis, IN) and FlowJo software (Tree Star, Inc.)). Forty % of the muscle samples (≈ 200 μl) were analyzed during acquisition. Cell counts were determined by the number of events within a given gate divided by the fraction of the sample analyzed (e.g., cell count/0.4). The cell counts were then normalized to the mass of starting muscle sample in grams. After excluding debris and doublets/clumps, white blood cells were identified as CD45+ cells. T cells were CD45+, CD3+. CD8 T cells were CD45+, CD3+, CD8α+. Conventional CD4 T cells were CD45+, CD3+, CD4+. Treg cells were CD45+, CD3+, CD4+, Foxp3+. Memory T cells were CD45+, CD3+, CD127+ (Rosenblum et al., 2016).

Histology

Muscle cross sections (8 μm thickness) were cut and mounted on glass slides with a cryostat at -27ºC then stained with Hemotoxylin and Eosin. Stained samples were viewed using an Olympus IX73 microscope at 4X magnification. The entire cross section was imaged using an Olympus SC50 color camera. The images were analyzed using CellSens imaging software (Tokyo, Japan) for evidence of necrotic/damaged muscle fibers as described previously (Koh and Brooks, 2001). Muscle fibers with cellular infiltrate, pale/discontinuous sarcoplasmic staining or with a swollen appearance were counted as necrotic/damaged fibers (Figure 3.3A). An investigator who was blinded to the experimental condition analyzed the images. The number of damaged fibers was expressed per muscle area.

T Cell Sorting and Adoptive Transfer

Two donor rats were used to harvest T cells for adoptive transfer. The DN (damage-naïve) donor rat remained unperturbed in the home cage prior to harvesting cells. The DE (damage-experienced) rat underwent the LC protocol (as described in *Muscle Torque Assessments and Lengthening Contractions)* two weeks prior to harvesting T cells. To collect T cells from the donor rats, they were deeply anesthetized using isoflurane and euthanized. After euthanasia, the spleen and inguinal lymph node (left side) was harvested and placed in ice-cold PBS with BSA (0.5%). The organs were crushed between two microscope slides, filtered (40 μm) and prepared for magnetic activated cell sorting (MACS) with the LS MACS separation columns and the midiMACS separator. T cells were positively sorted on the rat pan T cell antigen OX-52. Briefly, 1 x 10^8 cells were incubated for 15 minutes with Rat Pan T cell MicroBeads (Miltenyi Biotec, 1:5 dilution factor). Sorted T cells were cultured (5% CO_2, 37ºC) in RPMI medium for 2 to 6 hours.

Recipient rats underwent a bout of LC with pre and 5-minute post-LC torque tests. Immediately after the 5-minute post torque test, 2 x 10^6 T cells were suspended in 0.5 ml of sterile PBS and aspirated into a 1 ml syringe. To ensure intravenous injection, the tail was warmed in 40ºC water for 1 minute prior to inserting a 24 gauge IV catheter (Jelco) into a lateral tail vein. The needle was withdrawn and blood flowing into the catheter indicated intravenous placement. Finally, the syringe was attached to the catheter and the cell suspension was injected into the vein.

Statistical Analysis

A linear mixed models analysis was used to analyze these data. For the analysis of peak torque data fixed effects were Time, Group and the interaction between Time and Group. A rat identifier variable was included as a random effect and was nested with Group and crossed with Time. Torque data for bout 1 were used for SB3D rats and torque data for bout 2 were used for RB and cRB groups. A within-group analysis was done for the torque frequency data whereby fixed effects were Time and Hz and the rat identifier variable was crossed with both Time and Hz. To analyze damaged fiber histology data, the fixed effect of Group was nested with a rat identifier variable. A Tukey's HSD test was used for pairwise comparisons when a significant main effect was found. Normality of the data was determined by examining the residual by predicted plot and transformations were applied when appropriate. When comparing flow cytometry cell counts (cells•g^{-1} of muscle) the data were log transformed. Results were considered statistically significant when $p < 0.05$. Data are presented as mean ± SE unless otherwise specified.

Results

Study 1

The first purpose of study 1 was to assess the presence of the RBE and the cRBE in rats on the basis of muscle torque characteristics and histological evidence of muscle damage following damaging LCs.

Muscle Contractile Characteristics

A significant Group X Time interaction ($p < 0.0001$) was found for peak torque, whereby SB3D and cRB groups lost torque following LC with no recovery by 3-day post-LC Figure 3.2A). The RB group lost torque similarly from pre to 5 minutes post-LC, but restored peak torque by 3-day post-LC. In the SB3D group, peak torque decreased from pre-LC to 5 minutes post-LC (42.7 ± 1.6 to 18.6 ± 1.9 N•mm/kg, $p < 0.0001$) and did not recover by 3-day post-LC (19.6 ± 2.0 N•mm/kg, $p = 1.0$). A similar loss in torque was observed in the cRB group (pre: 42.8 ± 1.5, 5 minutes post-LC: 18.0 ± 0.9, 3-day post: 16.9 ± 1.4 N•mm/kg). The torque values in the RB group at pre and 5 minutes post-LC were not different than the SB3D or cRB groups (p-values ≥ 0.92), but peak torque at 3-day post (32.7 ± 1.8 N•mm/kg) was significantly greater than the 3-day post measurements in the SB3D ($p = 0.0001$) and cRB ($p < 0.0001$) groups. In both SB3D and cRB groups, muscle torque was reduced across the whole torque frequency curve at 5 minutes post-LC compared to pre (p values < 0.0001, Figure 3.2B). No recovery was observed by 3 days post-LC (all p-values > 0.5). In the RB group, 5-minute post-LC torque values were significantly less than pre (all p-values < 0.0001). However, by 3-day post-LC torque values departed from 5-minute post values at all frequencies greater than 20 Hz (all p-values < 0.04), and were not different than pre-LC values at stimulation frequencies up to 30 Hz (all p-values > 0.9).

Histological Damage Assessment

A significant effect of Group was observed for the number of damaged fibers/mm^2 ($p < 0.0001$, Figure 3.3B). There was no significant difference between SB3D and cRB groups (42.9 ± 6.8 vs. 34 ± 4.1 fibers/mm^2, $p = 0.78$). The number of damaged fibers was greatly reduced in the SB14D group (0.9 ± 0.2 fibers/mm^2) compared to SB3D and cRB ($p < 0.0001$), indicating that the muscle damage was largely repaired by 14 days post-LC. There were slightly more damaged fibers in the RB group compared to the SB14 group (2.7 ± .4 vs 0.9 ± 0.2, $p = 0.04$) but RB had many fewer than SB3D and cRB (both p values < 0.0001).

These data demonstrate that, on the basis of muscle torque characteristics and histological evidence, the RBE, but not the cRBE, is expressed in rats subjected to electrically stimulated lengthening contractions.

The second purpose of study 1 was to characterize the intramuscular T cell population following a single and repeated bout of LC. We hypothesized that the muscle T cell content following a repeated bout (in both RB and cRB) would be increased compared to a single bout of LC, and there would be an increased frequency of T cells expressing a memory phenotype (CD127+).

White Blood Cell Content

The muscle white blood cell (CD45+) content was used as a general assessment of inflammation (Figure 3.4). In the muscles of CC rats, 2.1 ± 0.5% of all cells were white blood cells (5.93 ± 1.4 x 10^3 cells•g^{-1}). The percentage of white blood cells in the SB3D group markedly increased to 63 ± 5.4% ($p < 0.0001$, 1.03 ± 0.20 x 10^6 cells•g^{-1}) of all cells. A similar increase over CC was observed in cRB (72.0 ± 6.2%, $p < 0.0001$, 1.57 ± 0.35 x 10^6 cells•g^{-1}) that was not different than SB3D ($p = 0.8$). SB3D and cRB also had a significantly greater percentage

of white blood cells than RB (8.5 ± 4.9%, $p < 0.0001$, 9.99 ± 6.52 x 10^4 cells•g^{-1},) and SB14 (3.2 ± 1.2%, $p < 0.0001$, 1.81 ± 0.06 x 10^4, cells•g^{-1}).

T Cell Quantity

Of the WBCs in nondamaged/noninflamed muscles (CC, SB14D, RB), 14.7 ± 2.4% were T cells (CD45+, CD3+). However, in damaged/inflamed muscles (SB3D and cRB) about 3.2 ± 0.3% of the white blood cells were T cells. In terms of T cells•g^{-1} (Figure 3.5A), there were significantly more in the exercised muscles of SB3D (2.54 ± 0.40 x 10^4, $p < 0.0001$), SB14D (3.15 ± 0.96 x 10^3, $p < 0.015$), RB (6.34 ± 2.45 x 10^3, $p = 0.0006$), and cRB (6.19 ± 1.37 x 10^4, $p < 0.0001$) compared to CC group (5.53 ± 0.79 x 10^2). SB3D and cRB muscles had more T cells compared to all other groups (all p-values < 0.01). We used CD127 as a marker for memory T cells. CD127 is elevated on memory T cells (including memory Tregs) in mice (Rosenblum et al., 2016). Memory T cells were scarce within muscle but tended to parallel the general changes in intramuscular T cells. In other words, the percentage of memory T cells remained around 3% among all exercised muscles with no significant differences. Memory T cells per gram of muscle were not significantly greater in RB (3.76 ± 1.74 x 10^2, $p = 0.5$) or SB14D (1.04 ± 0.64 x 10^2, $p = 0.7$) compared to CC (7.45 ± 3.0 x 10^1). There was no difference in memory T cells between SB3D and cRB (8.14 ± 1.70 x 10^2 vs. 1.34 ± 0.45 x 10^3, $p = 1.0$), but both SB3d and cRB were significantly greater than CC, SB14D and RB (p-values ≤ 0.04). Just as the memory T cells tended to mirror the general T cell content, CD4, CD8, and Treg subsets followed similar trends. In general, SB3D and cRB muscles had the greatest number from each subset, while RB and SB14 showed either a slight increase or no difference compared to CC. These data are represented in Figure 3.5B

Interestingly, T cells in the cRB group appeared to be higher than in the SB group. On average, cRB contained 6.19 ± 1.37 x 10^4 compared to 2.54 ± 0.40 x 10^4 T cells•g^{-1} in the SB group. These data were analyzed on the log scale due to nonhomogeneity of variance and the difference between them was not significant ($p = 0.17$). To illustrate the apparent difference between these groups, Supplemental Figure 1 (in the Appendix) shows these data on the linear scale. There was one influential data point that fell far below the mean in the cRB group. When this data point is excluded from the analysis, the difference between these groups is significant ($p = 0.03$). However, there was no indication that this data point should be removed due to measurement or experimental error.

T Cell Phenotype

The most plentiful T cell subset in the muscles that had done damaging exercise was the conventional CD4+ subset (SB3D: 68.9 ± 2%, SB14D: 61.0 ± 5.6%, RB: 57.6 ± 4.7%, cRB: 72.4 ± 8.7%). However, the conventional CD4+ subset contributed to a smaller percentage of the T cell pool in the CC muscle (23 ± 3.5, $p \leq 0.003$). The next most plentiful subset in the exercised muscles was the CD8+ subset (SB3D: 12.4 ± 1.6%, SB14D: 26.0 ± 2.2%, RB: 18.6 ± 0.5%, cRB: 13.5 ± 1.5%) The percentage of CD8 T cells was not different among groups ($p = 0.06$). Tregs (CD4+, Foxp3+) contributed a minor percentage to the T cell pool and did not differ among groups (SB3D: 7.1 ± 1.2, SB14D: 5.8 ± 1.3, RB: 6.5 ± 1.2, cRB: 8.0 ± 0.8, CC: 4.5 ± 0.9). Double negative T cells (CD8-, CD4-) contributed greatly to the T cell pool in CC muscle (43.3 ± 11.9), and this percentage was greater than other exercised muscles ($p \leq 0.003$). Double positive T cells (CD4+, CD8+) contributed a negligible percentage to the muscle T cell pool. These data are shown in Figure 3.6.

Spleen vs. Muscle T Cell Phenotype

The spleen T cell subset percentages were similar to those found in muscle that had done damaging exercise (SB3D, SB14D, RB and cRB), however the conventional CD4+ subset was relatively enriched in the SB3D and cRB muscles compared to the spleen ($p \leq 0.0004$), and CD8+ T cells contributed a larger percentage in the spleen compared to muscle ($p < 0.0001$). The percentage of double negative T cells was greater, and the percentage of conventional CD4+ T cells was lesser in CC muscle compared to the spleen ($p < 0.0001$). Muscle damage does not appear to greatly impact the T cell subset percentages in the spleen, as they were similar among groups (Group $p = 0.25$). See Figure 3.6.

The purpose of the adoptive T cell transfer study (Study 2) was to test the hypothesis that T cells from a rat that had previously sustained contraction-induced damage could confer protection against contraction-induced damage in recipient rats. Contrary to this hypothesis, rats that received T cells from a damage-experienced donor rat did not demonstrate any protection against muscle damage compared to rats that received T cells from a damage-naïve donor. Both groups lost a similar amount of peak torque from pre to 5-minute post-LC with no recovery by 3 days post-LC (Group X Time, $p = 0.25$, Figure 3.7C). The torque frequency analysis showed that muscle torque was reduced at all frequencies of stimulation at 5-minute post compared to pre-LC, with no significant improvement by 3-day post in either group Figure 3.7D. No difference in the number of damaged muscle fibers/mm^2 was found between naïve adoptive transfer and experienced adoptive transfer groups (29.5 ± 2.4 vs. 30.5 ± 2.1, $p = 0.5$, Figures 3.7A & 3.7B).

Discussion

Perhaps the first observation of rapid muscle damage-protective adaptation to strenuous exercise was published in 1963 (Highman and Altland, 1963). Now more than 50 years later,

strides have been made in our understanding of the nature of the RBE as well as its mechanistic underpinnings (Hyldahl et al., 2017). However, a firm and complete understanding of the mechanisms behind the RBE remain to be grasped. Here we investigate an inflammation-related hypothesis of the RBE focused on T cells. We have characterized the muscle T cell infiltrate using multicolor flow cytometry after a single bout of lengthening contractions as well as after a repeated bout of lengthening contractions done on the ipsilateral or contralateral limbs. We also carried out an adoptive cell transfer study to test the hypothesis that T cells contribute to the RBE in a way similar to their function in adaptive immunity.

This study confirms the presence of the RBE based on muscle contractile function and histological evidence of damage (Figures 3.2 and 3.3). The degree of force loss observed in this study is similar to previous studies in rats and mice using LC (Ingalls et al., 2004;Dipasquale et al., 2011), and similar to the typical strength loss seen in human upper limb muscles (elbow flexors (Paulsen et al., 2010;Chen et al., 2016)) following LC. There was no difference in the 5-minute post-LC torque measurements between SB3D and RB. However, the RB group restored torque by 3 days while SB3D exhibited no recovery. This observation, that damage-experienced rats are not protected from strength loss immediately after LC but display accelerated recovery in the following days, is the typical manifestation of the repeated bout effect in both rodents and humans (Ingalls et al., 2004;Hyldahl et al., 2015).

The myofiber necrosis observed here is consistent with other studies in rats and mice following lengthening contractions (Armstrong et al., 1983;Koh and Brooks, 2001;Dipasquale et al., 2011). That prior conditioning substantially reduces the frequency of myofiber necrosis by a bout of LC has been reported previously (Koh and Brooks, 2001), and the degree of protection appears similar to that presented here.

Contrary to our hypothesis, a previous bout of LC done on the contralateral limb (cRB group) offered no protection from damage (Figures 3.2 and 3.3). A summary of the literature on the cRBE concluded that it provides about 50% of the protection observed in the RBE (Hyldahl et al., 2017). As such, we expected that the cRBE animals would show evidence of damage resistance relative to SB3D, but not to the same degree as the RB group. Some studies suggest that the RBE is the result of neural adaptations that alter motor unit recruitment (McHugh et al., 1999;McHugh, 2003;Howatson and van Someren, 2007). However, some studies (including this study) have shown that the RBE can be realized in the absence of voluntary motor unit recruitment (Lapointe et al., 2002a;Ingalls et al., 2004;Corona et al., 2010;Dipasquale et al., 2011). However, this does not rule out the possibility that neural recruitment adaptations contribute to the RBE. It is believed that the RBE is not the result of a single adaptation, but rather multiple adaptions occurring together (Hyldahl et al., 2017). Therefore, it may be that some adaptations function only in the damage-experienced muscle (restricted category), while others are nonrestricted to the damaged muscle. As such, the "nonrestricted" adaptions may be effective in protecting damage-naïve muscles from injury. That the RBE is more potent and persistent than the cRBE (Chen et al., 2016) fits with this idea. The RBE might be more robust and long lasting because both restricted and nonrestricted adaptations contribute to it. On the other hand, the cRBE would be a product of only the nonrestricted adaptations. This study provides some novel insight into this supposition. To our knowledge, this is the first investigation that has tried to observe the cRBE using electrically stimulated contractions. Therefore, it is possible that electrically stimulated (nonvoluntary) contractions elicit only restricted adaptations, which are ineffective at protecting other damage-naïve muscles. Perhaps voluntary lengthening contractions are required to elicit nonrestricted adaptations (perhaps neural

recruitment adaptions) that are needed to realize the cRBE. Alternatively, it may be that the severity of the injury in the present study exceeded the protective capabilities of the cRBE. Most studies that have observed the cRBE were done in humans using voluntary LC, which causes a less severe injury than electrically stimulated LC (Crameri et al., 2007).

Because less damage is sustained after a repeated bout of LC, it seems logical that there would also be less inflammation. However, the data are mixed on this point. A previous investigation from our laboratory (Deyhle et al., 2015) showed that intramuscular proinflammatory chemokines, macrophages and CD8 T cells were increased after a repeated bout of LC compared to the initial bout. Other studies have also found that muscle inflammation is greater after a repeated bout of LC. Proinflammatory chemokine gene expression was significantly greater after a repeated bout of LC (Hubal et al., 2008) and intramuscular neutrophils increased after bout 2 but not bout 1 of LC (Stupka et al., 2001). Yet, other studies have shown the opposite. Macrophages and neutrophils accumulated in muscle following an initial bout of LC, however both cell types were significantly attenuated following a repeated bout (Lapointe et al., 2002a;Pizza et al., 2002). The discrepancy could be due to the method of contractions—those that showed attenuated inflammation used electrically stimulated contractions, while those that showed enhanced inflammation used voluntary contractions. Yet another study that used electrically assisted contractions observed a similar increase in macrophages following both bouts (Mackey et al., 2011).

In this study, inflammation was clearly attenuated in the animals that were resistant to damage (RB group). LC resulted in a large inflammatory response in the SB3D and cRB groups as evidenced by the substantial increase in intramuscular white blood cells (Figure 3.4). The white blood cell content in SB14D and RB was not different than the CC group, indicating that

inflammation was resolved by 14 days and that a second bout did not significantly increase inflammation. In parallel with the changes in total white blood cells, T cells (each subset measured, Figure 3.5) were increased in the SB3D and cRB groups but greatly reduced in the RB group.

To our knowledge, this is the first investigation to provide a detailed flow cytometric characterization of intramuscular T cells following LC. One earlier study using immunohistochemistry concluded that T cells do not infiltrate muscle following eccentric cycling exercise (Malm et al., 2000). Two other studies also using immunohistochemistry observed an increase CD8+ T cells in human muscle following damaging contractions (Marklund et al., 2013;Deyhle et al., 2015). One of these studies (Marklund et al., 2013) also did a separate stain for CD3. In that study, the number of CD8+ cells was only slightly lower than the number of CD3+ cells. This seems to indicate that most T cells in the muscle following damage were CD8+. Our results are contrary to this finding. Only about 13% of the T cells in the inflamed muscles (SB3D and SB14D) were CD8+ (Figure 3.6). Our data show that CD4 T cells, particularly the conventional subset (CD4+, Foxp3-), are especially responsive to contraction-induced injury. This subset was enriched in the muscle compared to the spleen (Figure 3.6). Another study found that only CD4 T cells and not CD8 T cells accumulated in the muscle following cardiotoxin exposure. Our results, which are consistent with others (Zhang et al., 2014;Fu et al., 2015), show that CD8 T cells do accumulate in muscle following injury, but contribute less to the T cell pool than CD4 T cells. Previous studies have shown that Tregs are particularly enriched in regenerating muscle following traumatic injury (Burzyn et al., 2013;Castiglioni et al., 2015), making up about 20% of the whole T cell pool at 3 days post-

injury (Castiglioni et al., 2015). We observed a lower percentage of Tregs (about 8 % of CD3+ cells) at 3 days post-LC (Figure 3.6).

We previously found that CD8 T cells were increased more after a repeated bout of LC when less damage was observed compared to the initial bout (Deyhle et al., 2015). This suggested to us that T cells were developing memory of muscle damage such that they were more responsive to a subsequent exposure. Given the necessity of T cells in effective muscle repair/regeneration (Burzyn et al., 2013;Castiglioni et al., 2015;Fu et al., 2015;Kuswanto et al., 2016), we suspected that the increased responsiveness following a repeated bout could indicate that they were promoting muscle repair more rapidly and thereby contributing to the RBE. However, if the T cells were responding to an initial and a repeated bout of LC in the same way that they respond to a primary and secondary immune response, we would expect them to be as or more numerous in the RB animals. The finding that T cells (all subsets) were attenuated in the RB group (Figure 3.5) and that the percentage of T cells expressing a memory marker (CD127 (Rosenblum et al., 2016)) was not greater in RB, cast doubt on the T cell memory hypothesis. Nevertheless, we carried out an adoptive cell transfer study to test this more directly. Adoptive transfer of T cells from muscle damage-experienced rats did not confer muscle damage protection (Figure 3.7).

While it appears that T cells do not promote the RBE by employing immunological memory, it remains possible that they and other immune cells play an active role in the development of the RBE. Blunting neutrophil (Lockhart and Brooks, 2008) or macrophage (Lapointe et al., 2002a) infiltration after an initial bout of LC hinders the acquisition of the RBE. Macrophages and neutrophils are major players in the muscle inflammatory response to damage. Here we have demonstrated that T cells are also responsive to contraction-induced damage.

Therefore, it is possible that T cells participate in the inflammatory responses that are necessary to bring about the RBE in a way that is not reminiscent of their role in adaptive immunity.

In conclusion, the RBE, but not the cRBE, is present in rats following electrically stimulated LC. A large inflammatory response is mounted which includes the accumulation of T cells in muscles not adapted to LC (SB3D and cRB). This population of T cells is particularly enriched with conventional CD4 T cells (CD4+, Foxp3−) but also includes CD8 T cells and Treg cells (CD4+, Foxp3+). Inflammation and T cell accumulation is attenuated following LC in damage-resistant muscles (RB). Because of the lack of T cell accumulation and the small population of memory T cells (CD127+) in the RB muscles, the T cell memory hypothesis is unlikely. Moreover, adoptive transfer of T cells from damage-experienced rats did not protect against muscle damage, which further strengthens this conclusion.

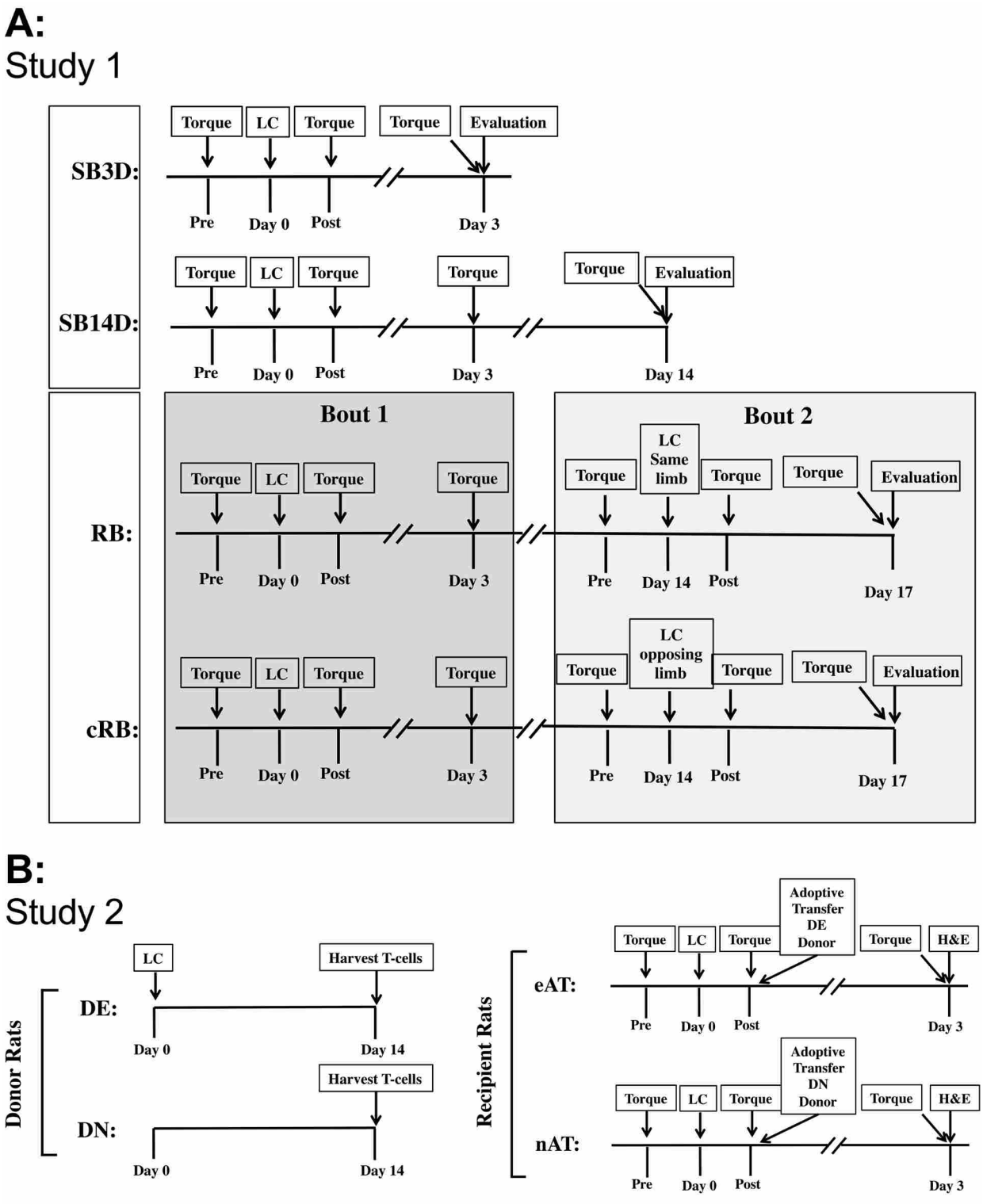

Figure 3.1 Study design schematics**. (A)** For study 1, 150 unilateral electrically stimulated lengthening contractions (LC) of the ankle dorsiflexors were used to cause muscle damage. In vivo muscle torque measurements were used to assess muscle damage. Rats were euthanized and evaluated (Evaluation) for T cell content using flow cytometry (muscle and spleen) and for histological evidence of damage (muscle only). Single bout 3-Day group (SB3D, n = 6) did a

single bout of LC. Single bout 14-Day group (SB14D, n = 3) did a single bout of LC with a torque assessment and muscle evaluation 14 days after bout 1. The Repeated bout group (RB, n = 6) did two bouts of LC on the same limb separated by 14 days. Contralateral RB (cRB, n = 6) rats also did two bouts separated by 14 days but the second bout was on the opposite limb. **(B)** For study 2, T cells were harvested from two donor rats. Damage-experienced donor rats (DE) did a bout of LC and T cells were harvested from the spleen and inguinal lymph node 14 days later. Damage-naïve donor rats (DN) did no damaging exercise prior to T cell harvest. Experienced adoptive transfer (eAT) rats received an intravenous injection of T cells from the DE rats immediately after a bout of LC. Naïve adoptive transfer rats (nAT) received T cells from DN rats. Muscle torque measurements were made to assess muscle damage. Tibialis anterior muscles were analyzed for histological evidence of damage using hematoxylin and eosin (H&E) staining.

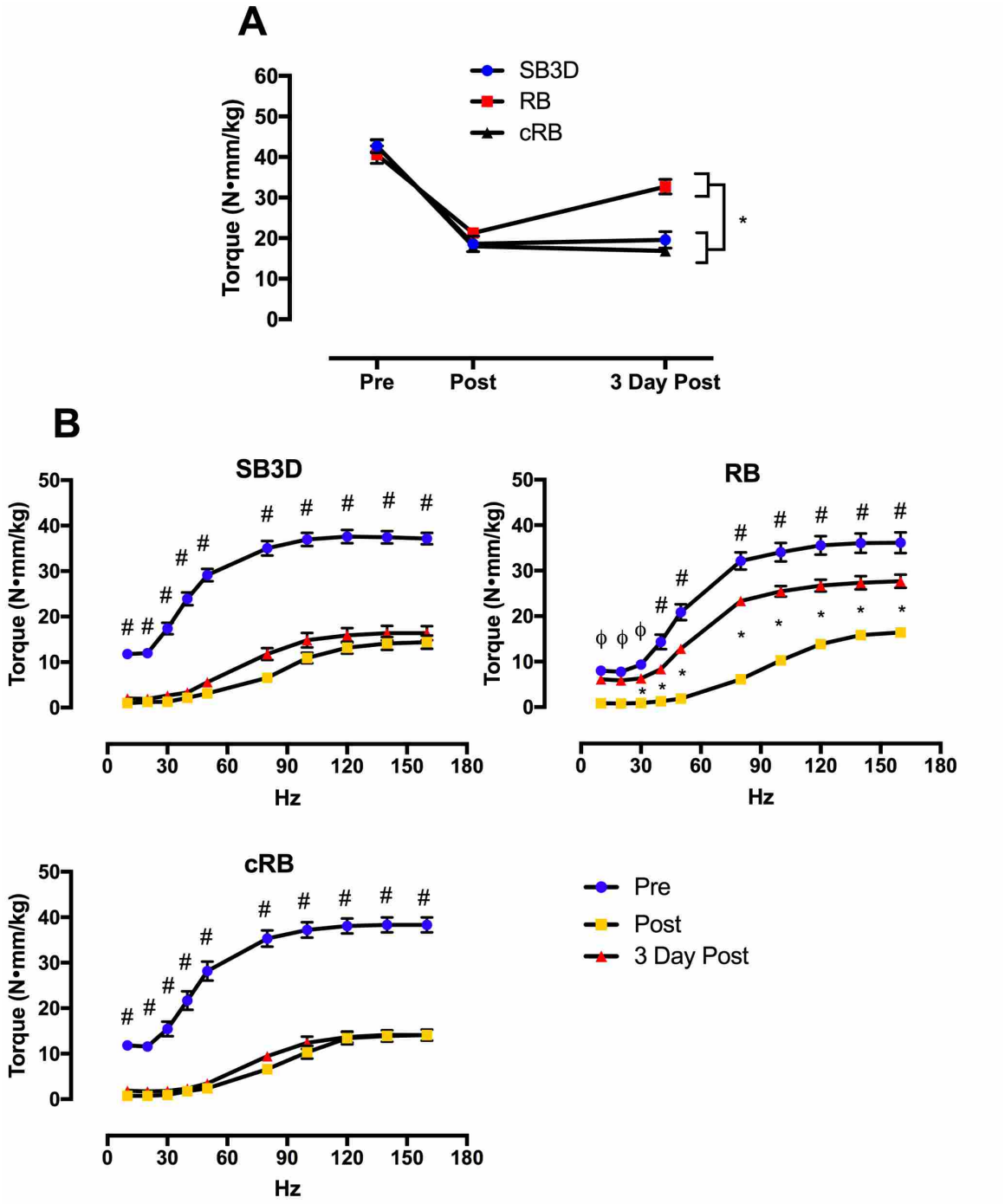

Figure 3.2 In vivo muscle torque characteristics in rats before and after damage. **(A)** Peak isometric tetanic contraction torque of the rat ankle dorsiflexors before (pre), 5 minutes after (post) and 3 Day Post 150 lengthening contractions (LC). * Indicates significant difference ($p < 0.05$). **(B)** Isometric torque as a function of stimulation frequency. SB3D did a single bout of LC. RB did 2 bouts of LC on the same limb separated by 14 days. cRB also did 2 bouts of LC separated by 14 days, but the second bout was done on the opposite limb. # Indicates different from post and 3-Day Post. ϕ indicates Pre different from Post. * Indicates 3-Day Post different from post. Data are mean ± SE. $\alpha = 0.05$.

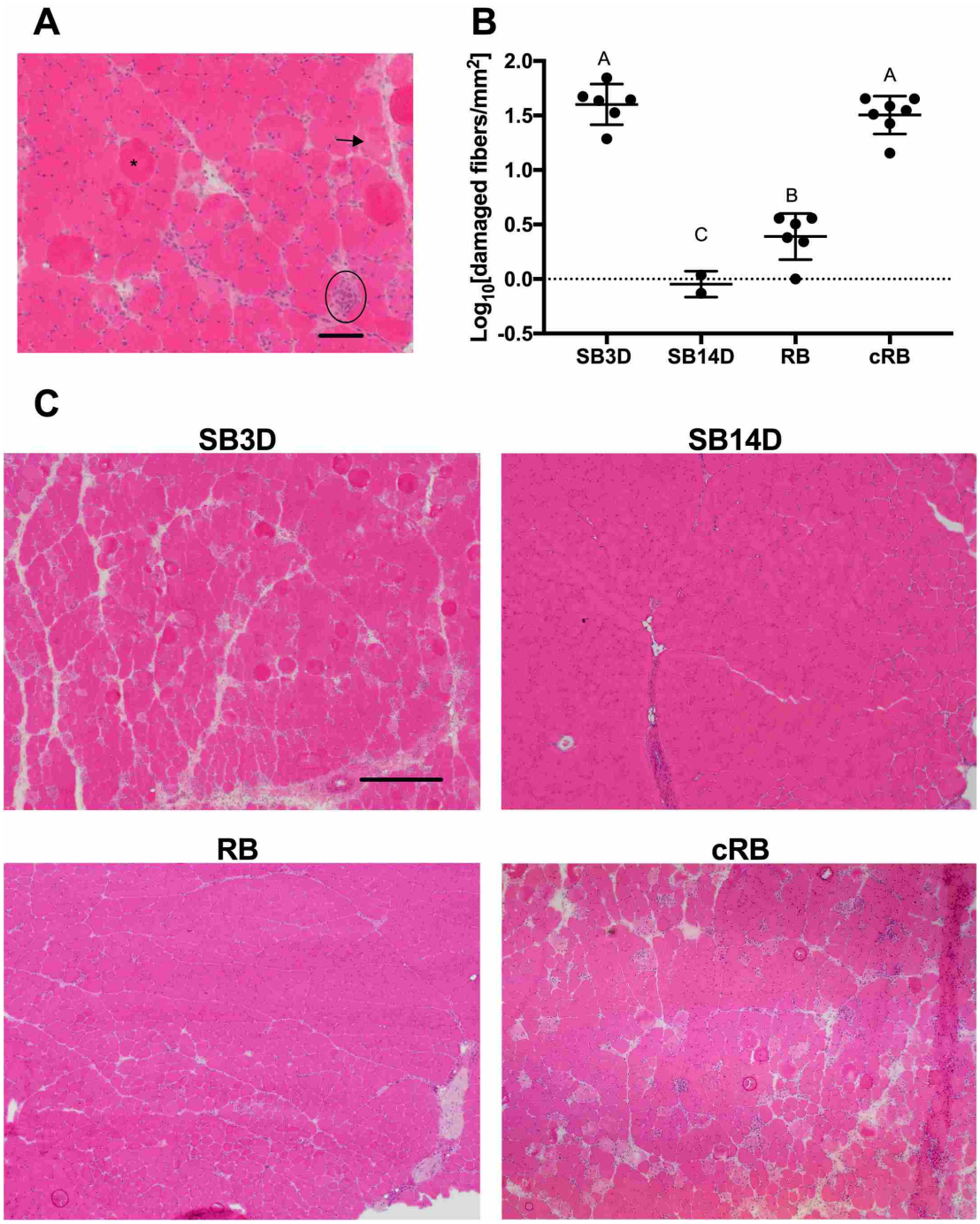

Figure 3.3 Muscle Histology following lengthening contractions (LC*).* This figure shows rat tibialis anterior muscle cross sections stained by hematoxylin and eosin (H&E) 3 days following a single bout of LC (SB3D), 14 days following LC (SB14D), 3 days after a repeated bout of LC (RB) and 3 days after a contralateral repeated bout of LC (cRB). **(A)** Muscle fibers with cellular infiltrate (circled fiber), pale/discontinuous sarcoplasmic staining (arrow), or a swollen appearance were identified as damaged/necrotic. Scale bar = 40 μm. **(B)** A quantification of the number of damaged fibers in each condition. Data are mean ± SE. **(C)** Representative images from each condition. Scale bar = 200 μm.

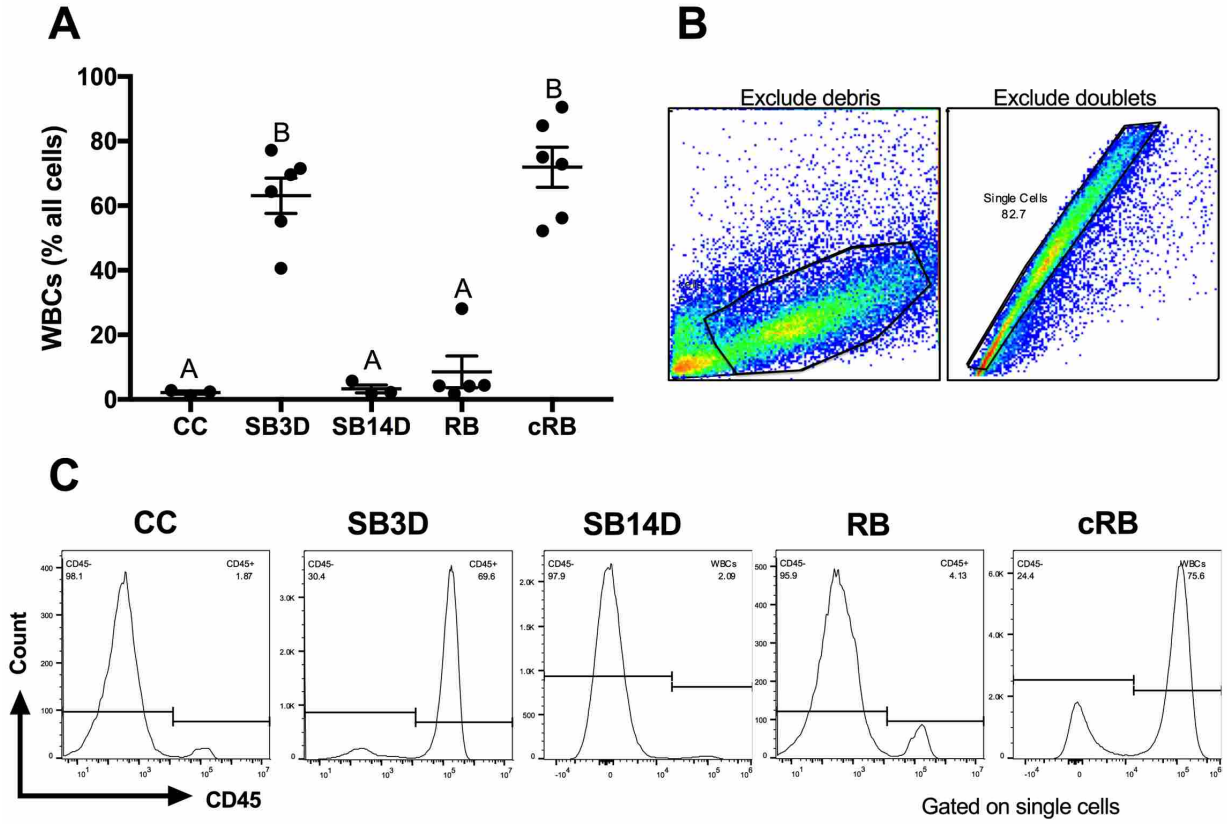

Figure 3.4 White Blood cells in muscle following lengthening contractions (LC). White blood cells (WBC, CD45+) were quantified using flow cytometry from rat tibialis anterior muscles after 150 lengthening contractions. **(A)** Intramuscular WBCs expressed as a percentage of all cells. Cage control (CC) underwent no contraction procedure. Single bout 3-Day rats (SB3D) completed a single bout of 150 lengthening contractions (LC) 3 days prior to analysis. Single bout 14-Day rats (SB14D) completed a bout of LC 14 days prior to analysis. Repeated bout (RB) rats did 2 bouts of LC separated by 14 days. The flow cytometry analysis was done 3 days after the second bout. Contralateral RB (cRB) rats also did 2 bouts, but the second bout was on the opposite limb and flow cytometry analysis was done 3 days post the second bout of LC. **(B)** The upstream gating strategy was to exclude debris and perform doublet discrimination. **(C)** Representative histograms for each condition.

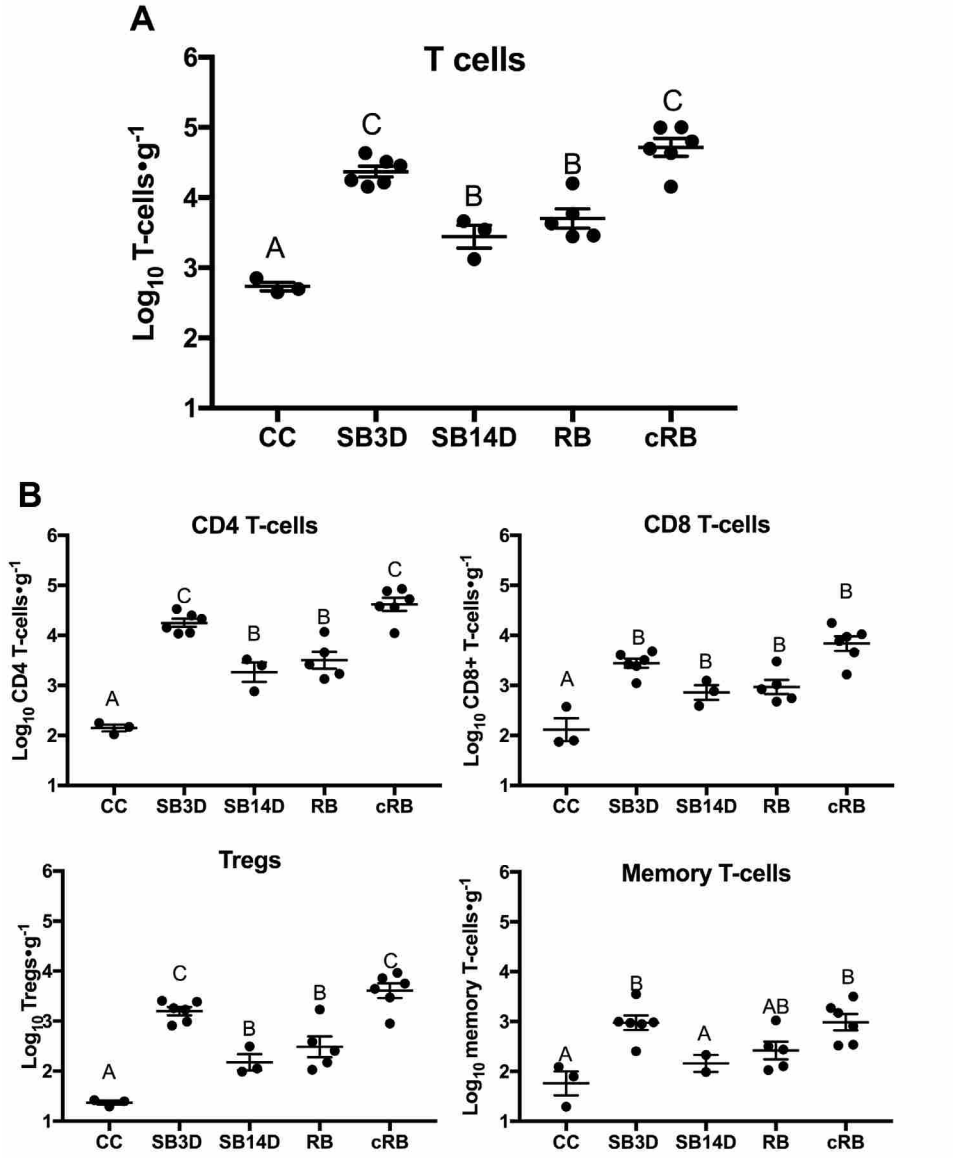

Figure 3.5 Intramuscular T cells after lengthening contractions (LC*).* T cells were quantified in the rat tibialis anterior following 150 LC. Cage control (CC) underwent no contraction procedure. Single bout 3-Day rats (SB3D) completed a single bout of 150 lengthening contractions (LC) 3 days prior to analysis. Single bout 14-Day rats (SB14D) completed a bout of LC 14 days prior to analysis. Repeated bout (RB) rats did 2 bouts of LC separated by 14 days. The flow cytometry analysis was done 3 days after the second bout. Contralateral RB (cRB) rats also did 2 bouts, but the second was on the opposite limb and flow cytometry analysis was done 3 days post the second bout of LC. **(A)** This panel shows the muscle T cell (CD45+, CD3+) content as a whole. **(B)** This panel shows the intramuscular content of 4 different T cell types. CD4 T cells (CD45+, CD3+, CD4+) CD8 T cells (CD45+, CD3+, CD8+) regulatory T cells (Tregs; CD45+, CD3+, CD4+, Foxp3+) Memory T cells (CD45+, CD3+, CD127+). Groups not connected by the same letter(s) are significantly different ($p < 0.05$) Data are mean ± SE.

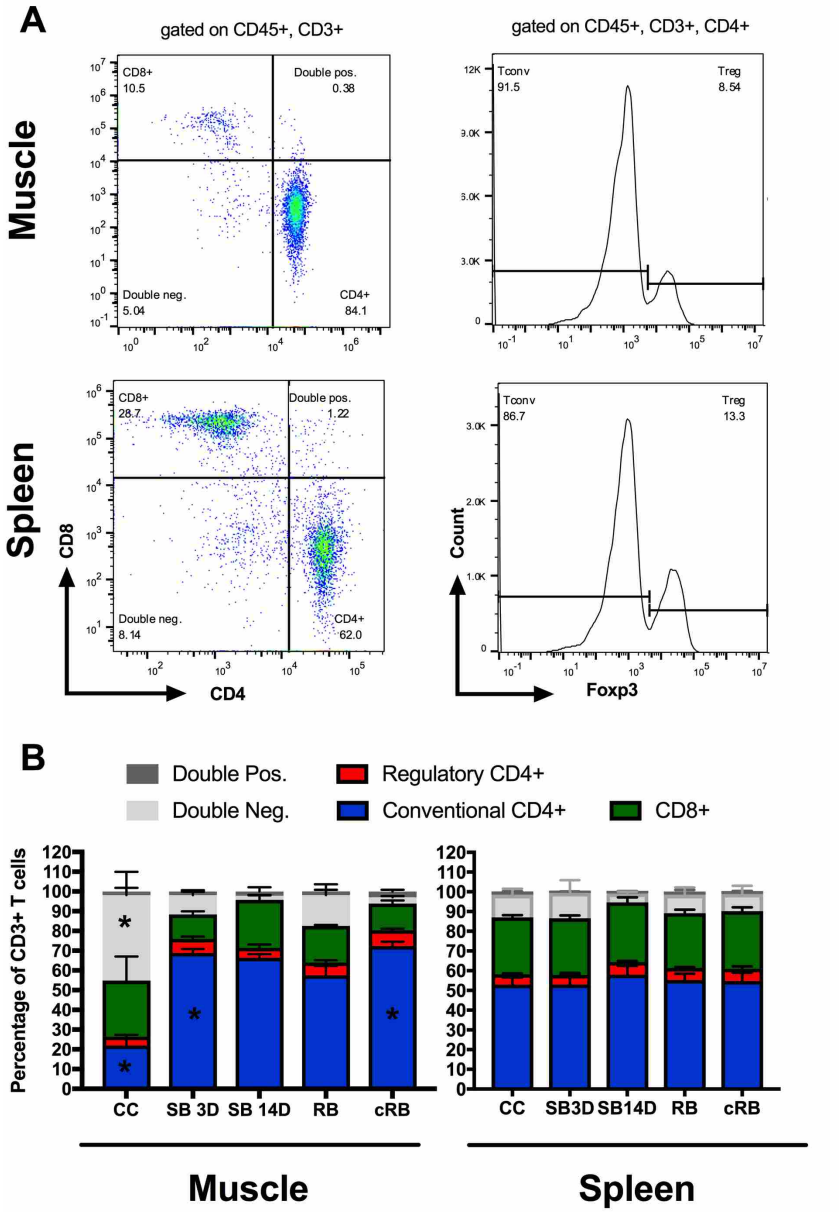

Figure 3.6 T cell subsets between injured muscles and the spleen. This figure shows the percentage contribution of rat T cell subsets to the whole T cell pool in muscle injured by lengthening contractions (LC) and the spleen. **(A)** Quadrant plot of CD8 vs. CD4 and a histogram showing Foxp3+ (regulatory CD4+) and Foxp3− (conventional CD4+) for both injured muscle and the spleen from the same rat (a rat from cRB group). **(B).** Quantified contribution of the T cell subsets to the whole T cell pool in the spleen and muscle of rats from 5 different conditions. Cage control (CC) underwent no contraction procedure. Single bout 3-Day rats (SB3D) completed a single bout of 150 lengthening contractions (LC) 3 days prior to analysis. Single bout 14-Day rats completed a bout of LC 14 days prior to analysis. Repeated bout (RB) rats did 2 bouts of LC separated by 14 days. The flow cytometry analysis was done 3 days after the second bout. Contralateral RB (cRB) rats also did 2 bouts, but the second bout was on the opposite limb and flow cytometry analysis was done 3 days post the second bout of LC. Data are mean ± SE. * indicates different from spleen ($p < 0.05$).

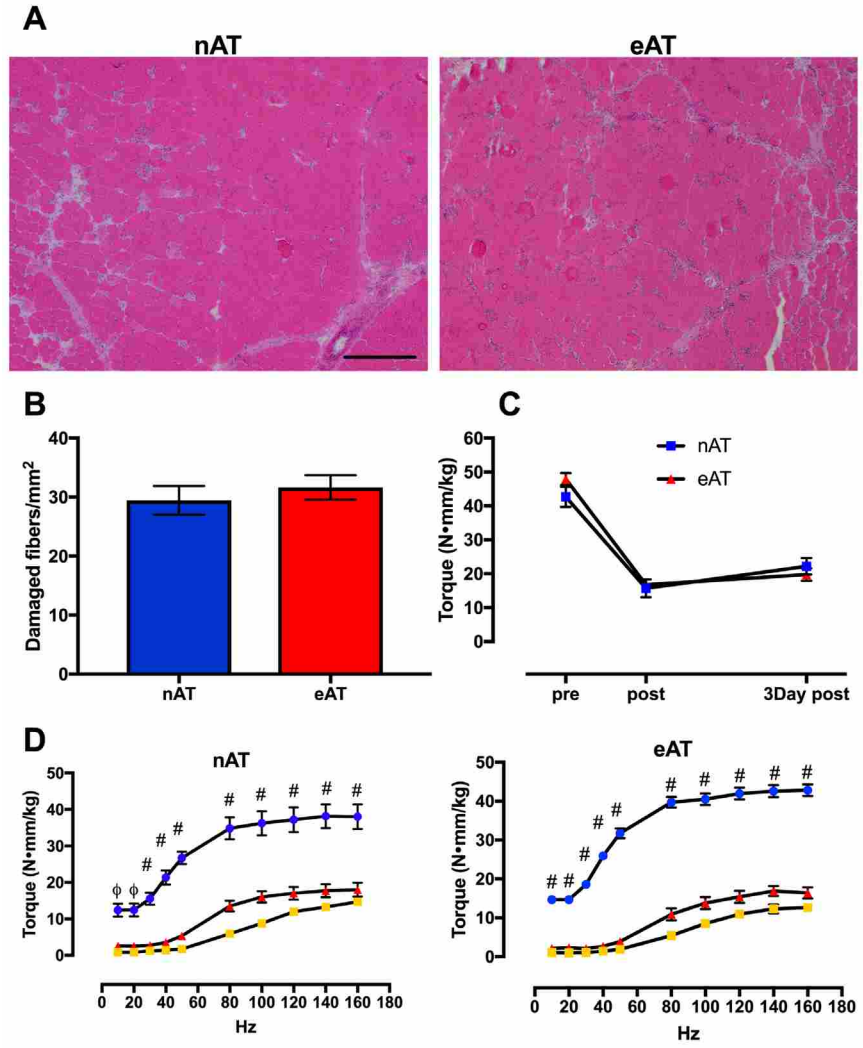

Figure 3.7 Adoptive T cell transfer and muscle damage following lengthening contractions (LC). Rats in the eAT (experienced adoptive transfer) group received T cells from a donor rat that had previously experienced LC. Rats in the nAT (naïve adoptive transfer) group received T cells from a donor that had not previously done LC. **(A)** Representative hematoxylin and eosin (H&E) images of muscle samples (tibialis anterior) 3 days after LC. Damaged muscle fibers are evident. Scale bar = 200 μm. **(B)** The number of damaged muscle fibers/mm^2 was not different between groups. **(C.)** Ankle dorsiflexor peak torque loss was not different between groups. * indicates different from pre ($p < 0.05$). **(D)** Muscle torque as a function of stimulation frequency was similar between groups. Blue dots = Pre-LC, yellow squares = 5-minute post-LC, red triangles = 3 days post-LC. # indicates different than 5-minute post and 3 days post-LC.

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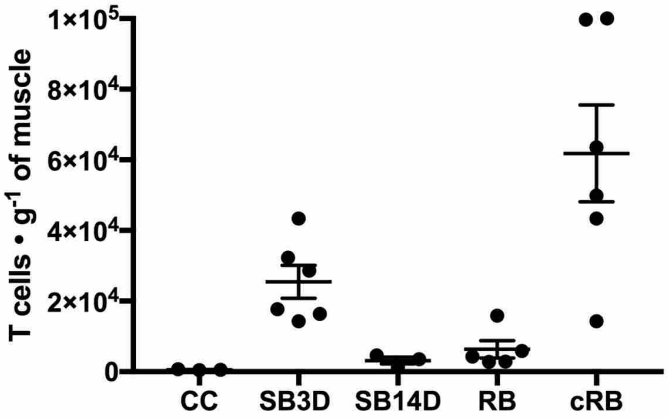

Supplementary Figure 1. Intramuscular T cell content following lengthening contraction-induced damage. T cells (CD45+, CD3+) were quantified from rat skeletal muscle by flow cytometry. Cage control (CC) underwent no muscle contraction procedure. Single bout 3-Day rats (SB3D) completed a single bout of 150 lengthening contractions (LC) 3 days prior to analysis. Single bout 14-Day rats completed a bout of LC 14 days prior to analysis. Repeated bout (RB) rats did 2 bouts of LC separated by 14 days. The flow cytometry analysis was done 3 days after the second bout. Contralateral RB (cRB) rats also did 2 bouts, but the second bout was on the opposite limb and flow cytometry analysis was done 3 days post the second bout. Note the high degree of variability within the cRB group.