2013

Immunomodulatory Effects of Massage in Skeletal Muscle

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IMMUNOMODULATORY EFFECTS OF MASSAGE IN SKELETAL MUSCLE

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Health Sciences
at the University of Kentucky

By

Christine Waters-Banker

Lexington, Kentucky

Director: Dr. Timothy Alan Butterfield, Professor of Rehabilitation Sciences

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2013

ABSTRACT OF DISSERTATION

IMMUNOMODULATORY EFFECTS OF MASSAGE IN SKELETAL MUSCLE

The inflammatory process is a critical component of the repair and regeneration of skeletal muscle following injury. The influx of innate immune cells following injury is intricate, and temporal nature. Although required for proper repair and regeneration, the inflammatory process has been shown to exacerbate initial damage, prolonging the healing process. Complementary Alternative Medicine (CAM) treatments, such as massage therapy, are a promising substitute for pharmaceutical modulation of the inflammatory response, and recent studies into the efficacy of massage have begun to report the physiological benefits of massage application following injury. Nonetheless, there is a significant lack of sound mechanistic investigations into massage application and its effects on unperturbed tissue. To gain insight to its potential influences on healthy skeletal muscle, massage was applied at three different magnitudes of load *in vivo.* Using a custom fabricated device for cyclic compressive loading, Wistar rats receiving massage had an increased expression in genes associated with the immune response; a significant change in the macrophage populations within the muscle tissue; and demonstrated a systemic effect marked by the increase of immune cells in the non-massaged limb. Further elucidating the systemic and immunomodulatory effects of massage, Long Evans rats receiving non-constrained eccentric exercise followed by a single 30minute bout of massage, displayed a significant crossover effect just 6 hours post exercise through the modulation of inflammatory cells in the non-massaged limb. Together these

investigations suggest that mechanotransductive properties of massage can promote modulation of the immune response absent of pharmaceuticals.

KEYWORDS: Massage, Immunomodulation, Skeletal Muscle, Macrophages, Inflammation

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For my Dad, Dale Waters, words can simply not express everything that you are and everything that you mean to me. Your example has shaped the person I am today. May this stand as a testament to all of your sacrifice and hard work.

Thank you for your love

ACKNOWLEDGEMENTS

I would be remiss if I did not give my appreciation and sincerest thanks to all of those who have helped and supported me in this venture:

Dr. Tim Butterfield for his incredible support and confidence. You embody what it is to be a mentor; I am truly fortunate and thankful for your guidance and friendship. Your thoughtfulness, support, patience, encouragement, and selflessness have had an incredible influence on my life. You have provided an example for which I aspire to become. I am forever grateful for the confidence you have in me, and the opportunities you have provided.

Dr. Esther Dupont-Versteedgen for serving as the chair of my committee, her dedication to my education, and the impressive example she sets for women in science. You have imparted invaluable wisdom to me beyond the walls of academia. I truly admire your confidence, work ethic, and successes throughout multiple facets of your life. You are an impressive force.

Dr. Patrick Kitzman and Dr. Karyn Esser for serving on my advisory committee and trying your best to focus my research aspirations. I now understand your purpose. It has been a pleasure to learn from both of you. Your wealth of experiences and accolades are inspiring.

Dr. Melinda Wilson for serving as the external examiner on my dissertation.

Sarah Abshire, for her steadfast patience and technical assistance. You have been my bench mentor, my confidant, my counselor, and friend. I have been so fortunate to work beside you these last four years. I could not have completed this without you.

Amy Confides and Margo Ubele for being the greatest neighbors to borrow a cup of sugar from… and antibodies…and Vectashield®… and whatever else we seemed to lacking.

Joythi Mula for imparting her infinite immunohistochemistry knowledge to me. You truly are a master.

Dr. Michael Reid for his generosity and wisdom. Although our meetings were usually brief they always left a lasting impression.

Dr. R. Grace Walton for talking me off the ledge and guiding me through my first RT-PCR. Your energy, honesty, and sense of humor are truly a breath of fresh air. You dance to the beat of your own drum and you rock at it.

Dr. Chris Fry, Dr. Janna Jackson, and Dr. Jonah Lee for treating me like a colleague, always making the time to entertain my questions, and letting me peer over your shoulders. You have been (and will be) great mentors.

Pandora® for lifting your 40 hour a month limit.

Kristie Bruner (Law) for keeping this place running and taking care of everything/everyone. I truly have no idea how you do it all, and still make the time to counsel us as well.

Dr. Mattacola for initially accepting me into the Master's program here at UK, providing me with opportunities to stick around, and throwing great bonfire get-togethers.

Nikki Lounsberry, for your support, encouragement, and our conversations over a great bottle of wine (or two).

My Grandpa and Grandma Waters, and my Aunt Melonie for attending all three of my graduations. I promise this was the last one. Your support does not go unrecognized, and it is greatly appreciated.

To my parents Jill and Dale Waters for your skilled child rearing. You have provided me with the wisdom and guidance that is directly responsible for the confidence, hard work ethic, and strong principals I have developed today. Your love and support is unmatched, and for that I am eternally grateful.

My brother Nick and sister Andrea Waters for your love and support. We may not be close in proximity but I but you are never far from my mind or my heart.

Achilles and Briseis your love and happiness bring me such joy and much needed stress relief. Thank you for dragging me to the dog park, it is truly therapeutic.

My husband Tyson, I can never thank you enough for embarking on this journey with me. Thank you for pushing me to succeed, for tolerating my crazy, for laughing at my jokes, for entertaining my weirdness, and always being there when I need you. This would not have been possible without you. Your love never ceases to amaze me. Infinity squared.

PREFACE

Chapters three and five, respectively, are based on the following manuscripts:

Waters-Banker C, Dupont-Versteegden EE, Kitzman PH, Butterfield TA (2012). Investigating the Mechanisms of Massage Efficacy: the Role of Mechanical Immunomodulation. *Journal of Athletic Training* (*in press;* December 2012; Publisher's ID: JAT0085-12R1).

Waters-Banker C, Butterfield TA, Dupont-Versteegden EE. (2013). Immunomodulatory Effects of Massage on Non-Perturbed Skeletal Muscle in Rats. *Journal of Applied Physiology* (*in revision,* Publisher's ID: JAPPL-00573-2013).

Waters-Banker C, Dupont-Versteegden EE, Butterfield TA. Crossover Effect of Massage following Eccentric Exercise Influences Abundance of Macrophage Populations at Six Hours. (*In progress* For submission to: *Journal of Physiology*).

This dissertation is based on a collection of stand-alone manuscripts, and therefore has some redundancy within the introduction and method sections of chapters three through five.

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CHAPTER ONE: INTRODUCTION

Skeletal muscle has a great capacity for repair, regeneration, and adaption in response to injury. Working in concert with the immune cells, the two systems coordinate an elaborate, and temporal response for the restoration of skeletal muscle tissue[1]. Depending on the severity of the injury, this process can take days to weeks to accomplish. Key mediators of this process are the cytokines that interact with the cells of the innate immune system: neutrophils, M1, and M2 macrophages. The ever changing microenvironment influences actions such as respiratory burst[2] and the release of cytotoxic chemicals (neutrophils), phagocytosis of necrotic tissue (M1) [1,3-6], and finally myoblast proliferation and satellite cell activation for repair and regeneration (M2)[3,5,7,8] . Though this is a highly orchestrated event, the plasticity that exists, particularly with the macrophage cell population,[1,9-11] provides exciting targets for the beneficial modulation of this process.

Current treatment of muscle injuries mainly include the use of non-steroidal anti-inflammatory Drugs (NSAIDs). NSAIDs provide effective pain relief due to their effective inhibition of the Cox enzyme.[12,13] Inhibition of Cox-2 in particular, plays a large role in the synthesis of prostaglandin E2 (PGE2) from the arachadonic acid released during damage of the cellular membranes.[12,13] Although blocking PGE2 prevents its ability to activate, and sensitize local afferent nerves, Cox-2 inhibitors have been shown to delay muscle regeneration by 20-33% up to three weeks following freeze injury in mice.[12] Interestingly, administration of Cox-2 inhibitors to healthy muscle does not affect healthy fibers, suggesting an important role for PGE2 in early muscle regeneration.[12] PGE2 has additionally been shown to have an influence on macrophage function. When produced by mesenchymal stem/stromal cells in co-culture with pro-inflammatory macrophages (M1), PGE2 appears to directly influence a phenotype switch to an anti-inflammatory macrophage (M2).[14] Together these data would suggest PGE2 as a potential early catalyst for the transition into the repair and regeneration process.

Therapeutic options that modulate the inflammatory response rather than inhibit components of it, would be most advantageous.

One such therapeutic modality is massage. An extremely ancient therapy, massage is currently sought after for the relief of numerous musculoskeletal problems. Positive effects of massage may be contributed to the mechanotransductive properties of cells that have the ability to transmit a mechanical signal into a biochemical response[15]. Muscle uses this property to constantly adapt to imposed demands over time. Through mechanotransduction, muscle can build sarcomeres in series and in parallel, as a response to overload and longitudinal stretch respectively[15-18]. When considering the intricate structure and interconnectedness that exists between muscle fibers, muscle has a remarkable potential to transmit mechanical force application through manual therapies like massage.

The pleotropic behavior of macrophages provides an attractive therapeutic target for manipulation. Through the manipulation of cytokine production, the change in environment could theoretically alter the phenotype of resident and/or infiltrating macrophages. Transitions that promote anti-inflammatory cytokine secretion could provide a beneficial microenvironment to promote repair and regeneration, while decreasing the opportunity for afferent nerve sensitization. Recent evidence[19,20] would suggest that an immunomodulatory component to massage does exist, and although its mechanism has yet to be elucidated, the macrophage could play an integral role.

Currently, investigations into the efficacy of massage are inconclusive due to the inherent variability that exists with massage applications. Reported effects are often subjective in nature, and the indications/contraindications for its use are not supported by sound scientific evidence[21]. Massage has maintained considerable popularity in the last 10 years as a complementary alternative medicine (CAM)[22-24], and is now becoming integrated in hospitals across the United States[25]. Currently the benefits of massage are vague, and the mechanisms unknown. Developing a better understanding of the immunomodulatory effects of massage, in addition to establishing appropriate parameters for its use will be

critical for its appropriate use. The potential for massage to modulate the inflammatory response could have far reaching influences of numerous treatments associated with the inflammatory response across various fields of health care.

The purposes of this dissertation were as follows:

- Develop a hypothesis driven model regarding the effects of massage on the inflammatory response in relation to infiltrating cells of the innate immune system, subsequent cytokine production, and the local afferent response through the concepts of mechanotransduction

- Evaluate the immunomodulatory effect of massage on non-perturbed muscle in relation to varying magnitudes of applied load

- To determine if a single bout of massage applied to eccentrically damaged muscle has the ability to modulate inflammation, creating a beneficial shift in the response over time, and whether or not systemic effect occurs.

The first purpose is addressed in chapter three. A review of the pertinent literature leads to the development of a hypothetical model, which, in part, will be tested in chapters four and five.

The second purpose is addressed in the fourth chapter. In which three different load magnitudes applied to non-perturbed tissue result in the modulation of the inflammatory response through cellular infiltration, and gene expression.

The third purpose is addressed in the fifth chapter. In which massage applied immediately following exercise resulted in a systemic effect, modulating macrophage cell numbers in the tissue of the non-massaged limb, and suggesting a shift in the inflammatory response.

The decision to utilize animal models for these experiments was based on previous literature[19,20,26,27], and the ability to maximize control of the treatment application.

Additionally, the use of Wistar, and Long Evans rats provided a variety of potential antibodies that have been established for the identification of immune cells[28].

CHAPTER TWO: LITERATURE REVIEW

2.1 Massage

2.1.1 History of Massage

Massage is a manual therapy that has been utilized for thousands of years to treat various musculoskeletal maladies, dating as far back as 2598 BC.[29] Ancient texts from Mesopotamia (present day Iraq) make reference to therapeutic procedures, akin to massage, including the application of liniments and tinctures by hand while rubbing the skin[30]. In ancient Greece, Hippocrates, discussed the concepts of massage in several writings and even Homer refers to "therapeutic rubbing" in his epic poem *The Odyssey*[31]. Massage was formally ingrained as a medical treatment when Hippocrates defined the treatment for conditions of pain, strains and injury in the *Hippocratic Collection*[32], leading Galen of Permagon, known as the "Father of Sports Medicine" to incorporate massage as a regular treatment for Roman Gladiators[33].

Although the popularity of massage died with the passing of the Roman Empire, its resurgence was brought about during the Renaissance era, and over time has eventually evolved into what we would consider modern massage today.[29] Massage has become one of the first manual therapies to be integrated alongside conventional medicine in hospitals around the US, sought after for its relief of pain, spasm, swelling, and its ability to provide relaxation.[25,34]

2.1.2 Massage as a CAM Therapy

Massage is encompassed within a vast range of non-traditional therapies termed Complementary and Alternative Medicine (CAM). CAM therapy consists of a variety of treatments that include dietary supplementation, chiropractic manipulation, and acupuncture[22-24]. Over the last 20 years CAM therapy has gained considerable popularity with annual costs reported upwards of $33.9 billion in the United States alone.[24] Those seeking massage therapy, usually do so in conjunction with conventional treatments, typically pay out-of-pocket expenses for "relief of symptoms" associated with musculoskeletal conditions, and chronic pain.[22,23] Additionally, a large number of

individuals report utilizing massage for wellness, disease prevention, and improved immune function.[35]

The most popular claim surrounding massage, is the thought that application increases local blood flow (hyperaemia), providing oxygen to promote tissue healing.[29,36-39] In cases of edema, massage is also thought to promote lymphatic uptake, reducing local swelling.[29,36-39] Scientific investigations into these claims have provided conflicting results utilizing numerous methodologies[36,38,39]. Although, massage may potentially increase local blood flow and lymphatic uptake, these effects are likely transient, and haven't been proven to be more effective than concentric contractions[37-39]. However, various strokes are applied to the skin to achieve these particular goals. Effleurage for example, is a unidirectional stroke that is applied from distal to proximal, along the axis of the tissue, with the intent to reduce muscle tone, and place the patient in a state of relaxation.[29] Whereas techniques like petrissage, consists of much more vigorous strokes such as folding, lifting, and even wringing fleshy regions of the body to stretch tissue contractions, and release fibrous adhesions.[29] Kneading involves slow circular movements that compress tissue against the underlying bone.[29] Contact is continuous as the practitioner moves proximally to promote the flow of tissue fluid, while relieving swelling and inflammation through the promotion of reflex vasodilation.[29] In contrast, techniques such as friction massage are applied perpendicular to the alignment of the underlying structures (muscle, tendon, or ligamentous fibers), and are intended to promote degradation, allowing for the realignment of fibers during the remodeling phase.[29]

To date, there are over 75 different methods associated with the application of massage[23] although strong scientific evidence for its efficacy is lacking. With a wide array of applicable techniques, pressures, and treatment times, massage is inherently difficult to control. This provides a certain level of difficulty when comparing results across multiple studies, often leading to inconclusive evidence[21], and anecdotal reports to corroborate massage as a beneficial treatment modality. Because muscle strain injuries account for 70% of all cases seen in a sports medicine clinic annually, sports massage is

becoming increasingly popular, and brings an increasing interest in uncovering the mechanisms that belie the reports of massage efficacy.

2.2 Muscle Strain Injury

2.2.1 Mechanical Mechanism of Muscle Injury

Although the exact mechanisms remain disputed, it is well accepted that repetitive active stretch (lengthening contractions or eccentric exercise) of skeletal muscle can result in muscle damage and delayed onset muscle soreness (DOMS)[40,41] Due to the force-velocity property of skeletal muscle, lengthening contractions exhibit greater tension per motor unit during activation. Thus, the high forces produced may overstress the activated fibers [42], and result in a mechanically mediated insult to the contractile apparatus (subcellular damage), or the muscle fiber membranes (cellular damage). Although this mechanical mechanism has been proposed to be the precipitating event during repetitive eccentric contraction induced muscle damage[43], the surface membrane appears to be resistant to mechanical disruption, even following stretches of extremely high, non-physiologic magnitudes[44,45]. Although alterations in sarcomere structure and organization have been reported immediately following one active stretch of single muscle fibers[46] there is no evidence that measurable subcellular damage is accompanied by an immediate disruption of the fiber surface membrane [44]. Remarkably, immediate surface membrane disruption in healthy muscle tissue has not been directly observed following lengthening contractions with physiologic strain magnitudes *in-vivo* [41]

In-vivo eccentric exercise protocols typically do not produce immediate severe cellular disruption, but instead result in sub-cellular damage of varying severity. Depending on the duration of exercise and the time frame of tissue analysis, lengthening contractions have been reported to result in various types of sub-cellular lesions. It has been shown that eccentric exercise *in-vivo* within the physiologic range of motion requires more than one stretch-shortening cycle to produce measurable muscle damage[47] due to fiber strains that are much smaller than those utilized *in-vitro*[48]. Although several human and unconstrained *in-vivo* animal models of repetitive eccentric exercise have shown severe fiber necrosis following eccentric-contraction biased exercise, tissues were analyzed

following hours or days of exercise [44,49]. There is evidence that surface membrane disruption following *in-vivo* exercise is a latent consequence of mechanical signal transduction, and mediated by an innate immune cell response[1,6,50,51].

2.2.2 Cellular Mechanism of Muscle Injury

Simple perturbations of skeletal muscle, such as an active stretch during eccentric exercise, have been shown to markedly increase the expression of TNF-α [52]. This pro-inflammatory cytokine is a neutrophil chemoattractant, and up-regulates the expression of endothelial-leukocyte adhesion molecules within the endothelium of the adjacent blood vessels [3]. Activation of the endothelium is site-specific and can result in the release of additional TNF-α, as well as additional pro-inflammatory cytokines including IL-1β, IL-6 and IL-8, all of which have been shown to attract inflammatory cells [1,53]

The traditional view has been that pro-inflammatory cytokines were released only by injured or damaged myocytes, resulting in the localization of neutrophils to these injured tissues. Thus, it has been argued that surface membrane disruption precedes the inflammatory response [54]. This has been supported by observations of neutrophil accumulation in muscle following eccentric contractions [55,56] and has even lead to speculation that damage-induced inflammation is the process responsible for delayed onset muscle soreness [57]. However, recently it has been shown that non-injurious isometric contraction and passive stretch can both induce diapedesis, with subsequent localization of neutrophils within the extra cellular matrix of skeletal muscle [5,7] illustrating that the early phase of the inflammatory response can precede membrane disruption.

Inflammatory cells play an important role in acute inflammation through removal of necrotic tissue or cellular debris and release of cytokines to modulate chemotaxis[8]. Neutrophils are the first subpopulation of white blood cells to enter traumatized or stressed tissue[58] and their main function is to contain and destroy damaged tissue or foreign matter through phagocytosis, respiratory burst, and degranulation[59]. Neutrophils generate hypochlorous acid via a muscle myeloperoxidase (MPO)-mediated reaction and

superoxide via NADPH oxidase to facilitate degradation and removal of damaged tissue. The potential for these cells to exacerbate muscle injury has been carefully studied in a variety of experimental models producing tissue damage through eccentric loading. For instance, observable alterations in cell structure following eccentric exercise have included the loss of sarcomeric organization, the loss of intermediate filament proteins including desmin,[60]; [61,62], and the infiltration of neutrophils within the muscle[5] ,[62]; [7]. This subcellular muscle damage is augmented through repetitive eccentric exercise, and further compromises the contractility and function of the muscle [47]. Although it has been accepted that neutrophils function in a contributory role to pre-existing muscle damage [4]it has been hypothesized that these inflammatory cells may provide the principal insult to the muscle membrane [55].

The majority of support for neutrophil-mediated myofiber damage comes from ischemia-reperfusion (I/R) studies where animals are rendered leukopenic using anti-neutrophil serum, anti-tumor agents, radiation [63 64], and may not serve as a good model for exercise induced muscle damage. Muscle can and does release fiber-derived cytokines or myokines that play a large role in the regulation and crosstalk between the muscle and the immune system[65]. These myokines can have both local and systemic effects as they can reach the circulation and affect various distant tissues[65]. Muscle cells themselves display receptors such as IFNγ IL-1, IL-6, and CCR2 that play an important role in the inflammatory response[65]. Chemokine C-C motif receptor 2 (CCR2) has been shown to play a critical role in the recruitment of macrophages directly from the bone marrow[65]. Lack of CCR2 receptors in muscle, severely hinder the repair and regeneration process[65,66]. It would appear that mechanical events are not necessarily, predisposing factor for membrane disruption, or responsible for the commencement of complex signaling sequlaes that result. The observed cellular destruction following eccentric exercise may instead be explained through an inflammatory cell-mediated response, that may respond differently to diverse situations.

2.2.3 Time Course of the Inflammatory Response

2.2.3.1 Neutrophils and Secondary Damage

Damage to the muscle fibers, and surrounding connective tissue, increases the expression of several pro-inflammatory cytokines. Cytokines are chemical cellular signals that influence the local microenvironment. Some key pro-inflammatory cytokines include: interleukin-1 beta (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8) and tumor necrosis factor-alpha (TNF-α)[1,67-70]. These cytokines stimulate the activation of endothelial-leukocyte adhesion molecules, P-selectin and E-selectin, which allow for the recruitment, adhesion, and infiltration of CD4 and CD8 T-helper cells, neutrophils, macrophages, and other effector cells via diapedesis.[1,3,68,71] Once these cells adhere to the vascular wall, they pass through gap junctions between the epithelial cells into the surrounding tissue, and migrate to various locations following chemoattractant gradients created by cytokines.[3,72]

Cell recruitment and subsequent migration to the site of damage is temporal in nature. Neutrophils are recognized as the first to respond, infiltrating the tissue as early as 1 hour, and typically peak from 6-24 hours.[1,3-8,55,73-75] The neutrophilic contribution to fiber degradation and/or repair is debatable, because the mere accumulation of neutrophils does not determine activation, or severity of injury.[76] Sensitive to their microenvironment[7,76], activated neutrophils are associated with the production of oxidants that can have detrimental effects on local tissues. Minute concentrations of arachadonic acid released from disrupted cell membranes, can act as both a chemoattractant and activator of neutrophils, initiating a respiratory burst releasing superoxide into the environment.[2] Muscles of neutropenic animals subjected to active stretch[75], as well as animals treated with antibody (M1/70) designed to prevent neutrophil respiratory burst[77], both demonstrate a reduction in myofibrilar damage. Together, these studies provide evidence favoring additional damage following neutrophil infiltration.

The chemicals produced by neturophils are incredibly cytoxic and non-selective to the break down of surrounding tissue[50]. Neutrophils themselves are highly susceptible to their environment and are usually short lived. Once activated they often spontaneously undergo apoptosis further releasing their contents into the enivironment[76]. This creates the production of free radicals and as a result they scavenge electrons from nearby cell

membranes compromising tissue integrity[76]. Once compromised, necrotic tissue can further the severity of the inflammatory reaction, producing additional pro-inflammatory cytokines, and attracting a larger number or pro-inflammatory macrophages to the area.

The neutrophilic response in skeletal muscle post exercise remains enigmatic, as their presence following damage is inconsistently reported in the literature [78]. Evidence exists that many of the surface receptors targeted by the antibodies currently utilized for the detection of neutrophils, are additionally displayed on the surface of pre-differentiated monocytes, macrophages, and dendritic cells as well[79]. Although, these cells are not considered homogeneous in nature, and in fact many of these cells have subtypes that can, carry out opposing actions[1,9-11,79]. This can make the identification of specific populations of innate cells very difficult to determine.

2.2.3.2 Macrophages

Following neutrophil invasion, circulating monocytes activate as they enter the tissue as phagocytic (M1) macrophages.[3,8] These macrophages are responsible for the removal of local debris, in addition to the production of numerous cytokines and growth factors that are thought to play a role in satellite cell activation and proliferation.[3,4,58,69,74,80-82] M1 macrophages are typically found at the injury site 12 hours post injury, peak around 24 hours, and sharply decline after 48 hours.[1,4,5,73] However, small numbers of M1 macrophages have been reported as early as 3 hours following freeze injury in rats.[81]

Non-phagocytic (M2) macrophages quickly replace the M1 macrophage population between 24-48 hours, and remain elevated for several days while the tissue remodels.[1,3-5,8,73] The activation of the M2 population following the conclusion of the phagocytosis, is recognized as the commencement of the regenerative process.[1] M2 macrophages produce various cytokines (IL-4, IL-10, Transforming Growth Factor-beta) and growth factors that have an anti-inflammatory effect, promoting tissue regeneration through wound healing, and the deactivation of M1 macrophages.[1] These macrophages are considered to be 'resident' macrophages and can be found throughout the tissue in the

epi- and perimysium during normal conditions; because of this, they are thought to be potential sensors of damage.[1,3,5,8]

However, the most incredible feature of macrophages is their plasticity. Macrophages have pleiotropic properties, and in response to their microenvironment, can undergo a phenotype transition.[1,9,10,14,51,83] Based on the local milieu of cytokines and growth factors, M1 macrophages can transition from pro-inflammatory M1 macrophages to anti-inflammatory M2 macrophages.[9,10,14] However, recent evidence in the last decade suggests that macrophage differentiation may not be terminal, and instead macrophages have the ability to continually evolve and alter their functions based on demands.[9,10,14] Further suggesting that their adaptation can even be reversible.[10]

Cultured macrophages incubated with different cytokines, either considered pro-inflammatory (interferon-gamma (IFN-γ), IL-12) or anti-inflammatory (IL-4, and IL-10), displayed different functional patterns when activated with lipopolysaccharide (LPS) a major cellular membrane component of gram-negative bacteria.[9] These functions continued to change as the cytokine environment changed from one to another.[9] Furthermore, specialized macrophages associated with certain cancerous tumors tend to resemble more of an anti-inflammatory phenotype (such as M2).[9] Treated with IFN-γ for 24 hours, and activated by LPS, these previously differentiated macrophages resembled a profile similar to that of a pro-inflammatory macrophage (such as M1).[9]

Macrophage engulfment of an early apoptotic, late apoptotic, or lysed tissue has been shown to play a role in functional cytokine secretion.[83] Consumption of lysed tissue by the M1 macrophage, promotes its continued release of pro-inflammatory cytokines such as TNF-α and IL-8.[83] However, if an M1 macrophage engulfs an early or late apoptotic neutrophil, they begin to change their functional pattern and secrete cytokines associated with anti-inflammatory M2 macrophages: TGF-β and IL-10, signifying a phenotype switch.[83]

Macrophage cytokine production following injury has a significant effect on the surrounding afferent structures as well. In fact afferent nerves have receptors that are specific to many cytotoxic chemicals released/created as products of the inflammatory response.[13,84,85] These nerves can be sensitized by any number of inflammatory mediators released following muscle injury including: adenosine triphosphate (ATP), TNF-α, bradykinin (BK), serotonin, prostaglandin E2 (PGE2), protons (H+) ions, and nerve growth factor (NGF). Once activated, afferents release neuropeptides such as calcitonin-gene related peptide (CGRP) and substance P (SP), further promoting edema through the dilation of adjacent vasculature.[13,84,85] Within hours, continual stimulation of the afferents can lead to increased density of sensory neurons in spinal cord (central sensitization), and within minutes an increase in receptive fields within the periphery.[13,86-88] This increase in nerve density both centrally and peripherally is likely due to the activation of dormant synapses.[13] This can lead to hyperexcitability and if persistent, create functional connections in the central nervous system.[13] Over production of NGF in particular, is associated with neuroplastic changes in the spinal cord that can lead to debilitating chronic pain syndromes.[89]

2.3 Massage and Mechanotransduction

2.3.1 Mechanotransduction and Skeletal Muscle

Cells are mechanosensitive structures, and therefore utilize the properties of mechanotransduction. Mechanotransduction is the transformation of mechanical stimuli into chemical cellular signals.[15,18,90] Mechanical deformation of tissues has the ability to trigger signaling events through an intricate cytoskeletal structure that consists of numerous mechanosensitive elements, such as stretch activated ion channels, and focal adhesion complexes. Signal transduction within and between cells can result in the alteration of protein expression.[15] It is well regarded, that muscle adapts in response to the stress it experiences. Most notably, adding sarcomeres in parallel to increase cross-sectional area (hypertrophy), as well as the addition, or subtraction, of sarcomeres when the muscle is chronically lengthened or shortened, respectively.[15-18]

Evidence has shown that cellular responses can be disparate within and between cells based on a given stimulus.[18] Experiments using C_2C_{12} myotubes cultured on elastic membranes were able to demonstrate that intracellular signaling differed in response to uniaxial versus multiaxial stretch.[18] Distinct responses as a result of stretch, suggest that signaling cascades are not random. Given the isovolumetric nature of muscle, this is an important concept to take into consideration where massage application is concerned. Manual compression or manipulation, may result in a negative strain in one location, but provide positive strains in other regions of the muscle. Muscle stiffness increases when load is applied at an angle to fiber orientation, altering sheer stress as fluid moves laterally against the plane of fiber orientation.[91,92] Multiple massage techniques, add to the intricacy of the resulting effects, emphasizing the importance of location, application, and direction of load administered.

2.3.2 Mechanotransduction and the Nervous System

Nervous tissue is mechanosensitive as well. Afferent nerve fibers (nociceptors) are located within the perimysium, adjacent to the vascular supply in muscles.[93] Afferent nerves (group III and group IV) are sensitive to noxious (tissue threatening) stimuli that exceed activation thresholds typically not activated during normal function.[84,94,95] When activated, the intensity of the stimulus is interpreted by the firing rate.[84,95] These fibers are classified by their mechanical threshold: either being low threshold mechanosensitive (LTM) or high threshold mechanosensitive (HTM).[85] When an injury occurs these thresholds can be altered often making them more sensitive to non-noxious stimuli. Increased firing rate is related to perceived pain in humans, however the actual concept of pain is processed at higher centers of the brain.[84,94,95]

Afferent nerves associated with muscle (typically group IV) have a relatively small diameter, and therefore a slow conduction velocity of about 2.5 m/sec.[96] Massage application is intended to stimulate larger diameter nerves that have faster nerve conduction velocities, thereby blocking afferent input (commonly known as the ‘gate control theory’ of pain management).[36] Furthermore, vigorous massage strokes are intended to cause some level of discomfort in order to activate descending pathways in

the central nervous system.[36] Descending pathways release endogenous opiates that lessen the pain transmitted to higher centers, theoretically decreasing perceived pain.[36]

2.4 Basic Investigations Regarding Massage

Recently, the fabrication of a novel massage device has allowed for fine-tuned, replicable load application to skeletal muscle *in vivo*. Using this device, massage has been shown to attenuate the inflammatory response, enhance functional recovery of force production[19], and other mechanical properties such as stress relaxation and creep[97] following eccentric exercise in rabbits. However, evidence also indicates benefits of massage are time-dependent, having a reduced effect the longer application was delayed after exercise. [20] Although each of these experiments significantly contributes to our understanding of massage benefits, the physiological mechanisms ultimately responsible for these results remain unknown.

A recent study[98] attempted to expose potential systems at the molecular level that may contribute to outcomes observed with massage. Human quadriceps biopsies subjected to massage following an eccentric exercise bout, showed a decreased expression in powerful pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), and interleukin-6 (IL-6) compared to control.[98] Together, these current works suggest mechanical manipulation to skeletal muscle has the potential to modulate the inflammatory response, limit damage following insult, and yield an advantageous environment for healing.

The attenuation of pro-inflammatory cytokines such as TNF-α and IL-6 could potentially influence the magnitude of the inflammatory response, and the subsequent infiltration of inflammatory cells. Muscle damage caused by mechanical perturbation, is dose dependent in nature, and will vary in extent to the regard of an inflammatory response.[5,7,99] Eccentric lengthening contractions are considered to be the most damaging, followed by concentric, isometric, and finally passive stretch.[7] In each instance circulating leukocytes and/or monocytes are almost immediately recruited to the site.[5,7,99]

This is the product of several pro-inflammatory cytokines and chemokines released into the environment that act as chemoattractants for these particular cells.

2.5 Summary

Current mechanistic investigations regarding the efficacy of massage have reported positive outcomes (e.g.) functional force recovery, which would suggest a modulation or attenuation in the inflammatory response. The potential for massage to alter the inflammatory response resulting in either: less detriment caused by innate immune cells, or a faster resolution of inflammation, could be of significant importance clinically for the treatment of inflammatory conditions. As such investigations into the immunomodulatory effects of massage could elucidate the potential mechanisms behind its action, and aid in the development of appropriate treatment parameters for optimal outcomes.

CHAPTER THREE: INVESTIGATING THE MECHANISMS OF MASSAGE EFFICACY: THE ROLE OF MECHANICAL IMMUNOMODULATION

3.1 Introduction

Complementary and Alternative Medicine (CAM) or sometimes referred to as Complementary Integrative Medicine (CIM), is steadily gaining popularity. CAM/CIM covers a vast range of treatments from dietary supplementation, to practitioner-based chiropractic and massage therapies.[22-24] Individuals seeking CAM/CIM treatments generally pay out-of-pocket costs that are comparable to family medical practitioner costs in a given year.[22,24] In 2007, the estimated costs were reported at $33.9 billion for CAM/CIM treatment in the United States alone, a third of which ($11.9 billion) stemmed from practitioner costs such as massage therapy.[24] Data have shown that the most commonly cited reason for seeking CAM/CIM therapy, such as massage, is for the treatment or prevention of musculoskeletal conditions, or those conditions associated with chronic pain.[23] The majority of individuals that utilize CAM/CIM modalities do so in conjunction with traditional treatment, citing "relief of symptoms" as one of the most common reasons.[22]

Previous investigations regarding efficacy of massage have proven to be highly variable and inconclusive. This is most likely due to inherent challenges associated with investigating the effects of massage such as inconsistent modes of massage application (i.e. effleurage vs. petrissage, or a combination of the two), applied forces, and duration of reported massage application. Although, these studies do attempt to mimic the clinical setting, the variability that exists between and within these investigations, makes them difficult to interpret as was recently concluded in a systematic review.[21] Additionally, many of the previous studies relied exclusively on subjective participant outcomes [21], and therefore conclusions about cellular and molecular responses are absent.

Considering the large number of individuals receiving CAM/CIM therapies, and the purported positive health benefits these modalities provide, the purpose of this communication is to explore how massage affects inflammatory response and the

modulation of pain. Beginning with an overview of the inflammatory response, readers will gain an in-depth understanding of immune cell function, and how endogenous chemicals released in this process effect pain transmission through the sensitization of afferent nerve fibers. Next, this communication will introduce the concept of mechanotransduction, and its importance in stimulating cell-signaling pathways. Finally, immunomodulatory effects of massage will bring all of these elements together to discuss the physiological benefits of massage application following injury. A better understanding of the physiological consequences induced by massage on cellular mechanisms underlying inflammatory pathways and pain modulation, will allow clinicians to make informed decisions about treatments associated with musculoskeletal injuries.

3.2 The Inflammatory Response to Muscle Injury

Injury to skeletal muscle is associated with sequelae of inflammatory events, and a sound understanding of the temporal nature of the immune response is necessary to provide effective treatment. Immediately following injury, skeletal muscle is rapidly invaded by several distinct populations of immune cells (monocytes) in response to abundant fluctuating signals regulated by the local tissue.[1,3-8,55,73-75] Increased expression of several pro-inflammatory chemical cellular signals known as cytokines (i.e. interleukin-1 beta, interleukin-6, interleukin-8, and tumor necrosis factor-alpha) stimulate the activation of endothelial-leukocyte adhesion molecules P-selectin and E-selectin.[1,3,68] In combination with various chemoattractants, cytokines take part in the activation of the CD4/CD8 T-helper inflammatory response, promoting the recruitment, adhesion, and infiltration of neutrophils, macrophages, and other effector cells via diapedesis from the vasculature into the surrounding tissue.[71]

Cytokines are released into the environment from multiple tissues such as the muscle cells, local resident macrophages, and mast cells. The shifting local environment promotes dendritic cells (specialized immune cells found in the blood stream and tissue) to travel to neighboring lymph nodes.[67,100] Once there, dendritic cells influence differentiation and mobilization of T-helper cells (T-cells) and B-cells to the site of injury

or infection based on the demands of the environment (**Figure 3.1**).[67,100,101] The cytokine expression determines the differentiation of reacting macrophages to their respective cytokine linage, either Th_1 or Th_2. These macrophages are categorized as either the "classically activated" macrophage (M_1) or the "alternatively activated" macrophage (M_2).[1,67,100,101] Cytokines of the M_1 phenotype stem from the Th_1 differentiated cell line and are considered pro-inflammatory. [67,100,101] Those associated with the M_2 phenotype derive from Th_2 differentiated cells and are considered anti-inflammatory in nature.[67,100,101] Additional T-helper cell pathways have been identified, but for the purposes of this communication, the focus will remain on the well-established Th_1/Th_2 taxonomy.[100]

3.2.1 Neutrophils

Cytokine signaling is an essential and influential factor driving the inflammatory response. Swift infiltration of neutrophils into the area is due to the activation of the Th_1 cytokine pathway, which in turn is an important process in inflammation.[1] Activation of the Th_1 cytokine pathway is influenced by the release of established neutrophil attractants that have been shown to be up-regulated immediately following electrical stimulation in myotubes in vitro.[1,102,103]

It is well recognized that invading neutrophils are the first to arrive to the site of damage following tissue injury, [1,3-8,55,73-75] and produce tissue damage through what is referred to as respiratory burst. Arachidonic acid is a structural component of cell membranes and is released with tissue disruption.[2] Arachidonic acid is not only a chemoattractant for neutrophils, but a stimulatory agent of respiratory burst as well.[2] Small concentrations of arachidonic acid, as little as 5μM, can initiate a respiratory burst in which superoxide is released into the environment.[2] Neutrophils exacerbate the breakdown of tissue through lipid peroxidation, leading to free radical release within the environment.[8,55] Free radicals scavenge electrons, stealing them from the lipid membranes of surrounding cells starting a chain reaction of free radical release.[8,55] As arachidonic acid concentrations increase, the local environment greatly influences the respiratory burst of neutrophils in a dose dependent manner, ultimately increasing the initial tissue damage.[2]

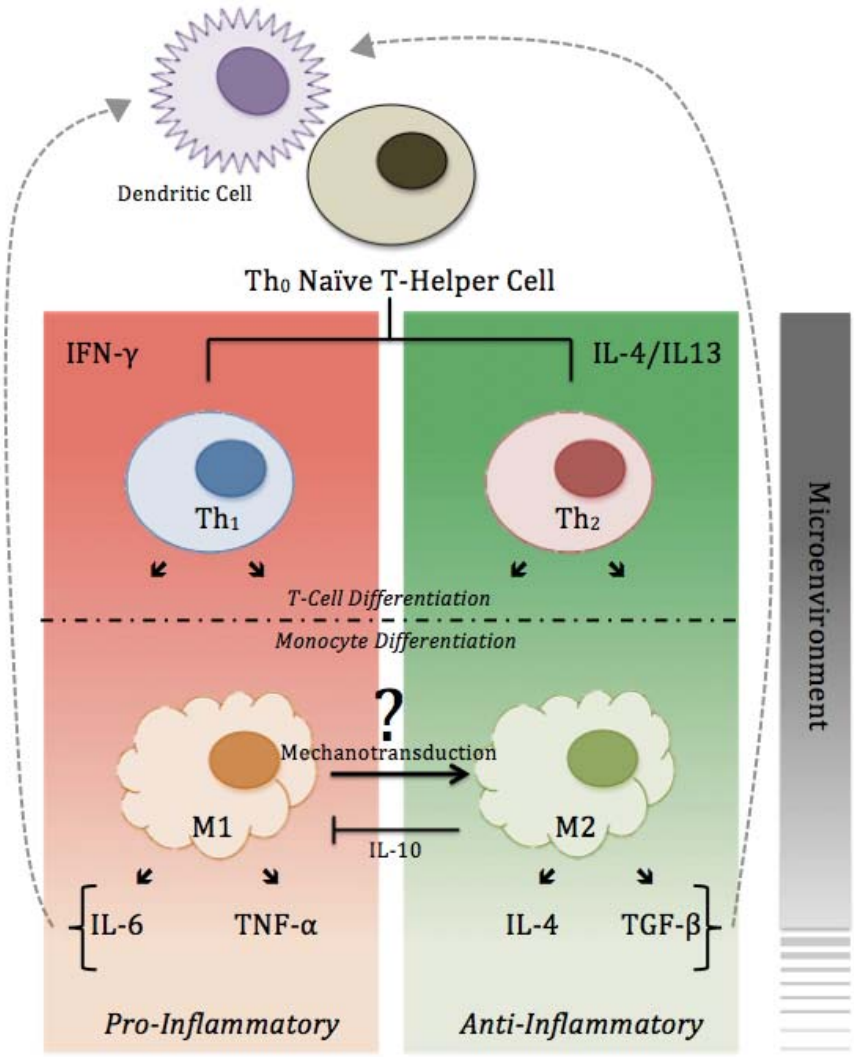

Dendritic Cell
Th0 Naïve T-Helper Cell
IFN-γ
IL-4/IL13
Th1
Th2
T-Cell Differentiation
Monocyte Differentiation
?
Mechanotransduction
M1
M2
IL-10
IL-6
TNF-α
IL-4
TGF-β
Pro-Inflammatory
Anti-Inflammatory
Microenvironment

Figure 3.1 Hypothetical model for the influences of massage on macrophage phenotype (Continued):

Simplified depiction of the cytokine linage of M_1 and M_2 macrophages within their respective pathways. Monocyte polarity is not only influenced by the dendritic cell/T-helper cell interaction, but largely through the local microenvironment in which they invade. In this figure, the pro-inflammatory environment consists of cytokines interferon-gamma (IFN-γ), interleukin-6 (IL-6) and tumor necrosis-alpha (TNF-α). Input from the dendritic cell as well as the microenvironment environment, aids the Naïve T-Helper Cell (Th_0) differentiate into a T-helper cell of the T-helper 1 (Th_1) pathway. This Th_1 cell will now further promote a pro-inflammatory influence on macrophage differentiation creating an abundance of M_1 macrophages, which in turn produce IL-6 and TNF-α into the microenvironment continuing the cycle. However, this plastic differentiation can allow a phenotype transition based on environmental demands. Manual therapy may allow for beneficial manipulation of this environment through the properties of mechanotransduction. In the event of a phenotype change from an M_1 to an M_2 macrophage, anti-inflammatory cytokines, interleukin-4 (IL-4), transforming growth factor-beta (TGF-β), and interleukin-10 (IL-10) will cause a shift in the microenvironment, influencing local dendritic cells to alter the T-helper differentiation to the Th_2 pathway through IL-4 and interleukin-13 (IL-13). Th_2 cells can then instead promote macrophage differentiation creating an abundance of M_2 anti-inflammatory phenotype, signifying the repair and regeneration phase.

Neutrophil production and release of oxidants has detrimental effects on the surrounding tissue creating myofibrillar damage in muscle. Utilizing antibodies that prevent neutrophil infiltration and respiratory burst in damaged tissue (such as M1/70) result in the reduction of myofibril damage.[77] Similarly, when applying an active stretch to the tibialis anterior muscle of a neutropenic rabbit (animal lacking or having severeished levels of circulating neutrophils), significant reductions in cellular damage have been reported 24-hours post exercise when compared to healthy control rabbits.[75] Together these studies indicate that a large amount of cellular disruption is due to the inflammatory process, and neutrophils specifically. This is further supported by evidence showing that the mere presence of neutrophils in the extracellular spaces of muscle is not damaging if they do not undergo a respiratory burst.[77]

Although the destructive, oxidative nature of neutrophils within the inflammatory process seems obvious, the beneficial functions of a neutrophilic response are less apparent. This is because the benefits are derived from the ability of the neutrophil to contribute significantly to subsequent macrophage activity. For example, neutrophil-macrophage interaction was demonstrated when skeletal muscle injected with snake venom regenerated at a much slower rate in the absence of neutrophils when compared to control muscle.[104] This attenuated regeneration was likely due to altered macrophage function, resulting in an actual delay of their recruitment to the damaged area.[104] This suggests the tissue environment, and principally: cellular signaling, as a target for selective manipulation for regulating the immune response in muscle.

3.2.2 Macrophages

Following neutrophils, macrophages are second to arrive on site, typically peak around 24 hours post injury, and remain elevated for several days.[1,4-6,8,55,73] Macrophages have a predominant role in the repair and regeneration process of muscle tissue, and are an excellent source of growth factors. Macrophages are responsible for the secretion of over 100 different chemical factors including established chemoattractants for inflammatory cells.[3,58,69,74,80] Macrophages are divided into two subpopulations (M_1 and M_2) that exhibit a disparate specificity of function in the immune response.

M_1 are circulating monocytes that become activated as they invade tissue.[3,8] The main role of M_1 macrophages is to phagocytose necrotic tissue.[3-5] The M_1 population has not been shown to directly cause damage to surrounding tissue, but they may interact with neutrophils to cause cell destruction indirectly.[1] M_1 macrophages enter the tissue from the vasculature approximately 24 hours post-injury, but sharply decline in number around 48 hours after injury when they are replaced by non-phagocytic M_2 macrophages.[1]

Macrophages of the M_2 population are referred to as 'resident macrophages'.[3,5] These macrophages exist throughout the muscle tissue and are thought to be potential sensors of damage [3,8] although, they are not thought to 'activate' until the process of phagocytosis has ceased. The M_2 macrophages are further divided into three additional subpopulations consisting of three categories: M_2a, M_2b, and M_2c. Each macrophage subpopulations is activated by a different set of cytokines that, in turn, release specific signals to promote tissue regeneration through: 1) wound healing and tissue repair, 2) anti-inflammatory responses, and 3) deactivation of the M_1 macrophages, respectively.[1] Activation of the M_2 population coincides with the commencement of the regenerative process [1], contributing to tissue repair through myoblast proliferation and satellite cell activation.[3,5,7,8]

A unique characteristic of macrophages is their high capacity for plasticity. In response to environmental demands, macrophages can undergo a phenotype transition shifting from a M_1 population to a M_2 population [1,67] (**Figure 3.1**). In terms of the inflammatory response, promoting an anti-inflammatory condition with a higher concentration of M_2 macrophages versus a pro-inflammatory M_1 dominated environment may be desirable. Several avenues are available to modulate the inflammatory response: arachidonic acid release, neutrophil recruitment, limiting the respiratory burst, or promoting an early macrophage phenotype transition.

Macrophages are considered malleable because of their ability to adapt to their local surroundings, and become biased by well-orchestrated signaling mechanisms (both intra- and extra-cellular in nature). Because of their malleability, inflammatory signaling cascades present a logical avenue for manipulation. Through the use of modalities like

massage, clinicians can modulate damage following injury, and restrict uninhibited secondary injury. Because M_1 macrophages are dependent on neutrophil action, an attenuation of neutrophilic response will reduce both lipid peroxidation and M_1 recruitment.[3,5,6,8] Theoretically, this could lead to early tissue repair and regeneration via the M_2 subpopulation. Or perhaps massage may promote prompt macrophage phenotype transition of M_1 to M_2 through tissue manipulation. Like other immunomodulatory interventions that are more common and empirically tested (pharmacological, thermal modalities), manual therapy holds great potential for its ability to modulate the immune response. (**Figure 3.1**).

3.3 The Afferent Nerve Response to Muscle Injury

As CAM/CIM modalities such as massage are often sought out as a treatment for chronic pain, the afferent nerve response to inflammation deserves mention. It is important to note that the actual concept of pain is one that involves numerous factors that are processed at several higher centers of the brain, including the linkage of an emotional response. At the tissue level, perceived pain in humans is related to the increased firing rate of nociceptors.[84,94,95] For this particular discussion, we will focus on the acute aspects of musculoskeletal injury in regards to nociceptive activation.

Muscle fibers themselves contain no afferent nerve endings within the confines of their cell membranes. Instead, afferent nerves are located in the perimysium surrounding muscle fascicles, adjacent to the vasculature that serves as the entry point for various immune cells.[93] These nerves are sensitive to noxious (tissue threatening) stimuli that, when strong enough, will elicit an action potential whose intensity is interpreted by firing rate.[84] These nociceptors have varying activation thresholds, and therefore are not activated during typical functional movement.[84,94,95] Afferent nerve fibers are classified as either low threshold mechanosensitive (LTM) or high threshold mechanosensitive (HTM).[85] In the event of an injury these thresholds can be altered making them increasingly sensitive and more likely to depolarize.

Afferent fibers also consist of numerous receptors that are sensitive to endogenous chemicals released during injury via the disrupted muscle and/or the inflammatory

cells.[84,85] Nerve fibers have receptor sites that are sensitive to bradykinin, serotonin or 5-Hydroxytryptamine, prostaglandin E_2, adenosine triphosphate (ATP), and histamine.[84,85,94,95] These have all been established as nociceptive stimulants, and are identified as being released from muscles when cell membranes are disrupted in response to injury. These substances can have long lasting effects, often potentiating one another.[85]

Neuropeptides are released from the nerve itself during the inflammatory response, and include substance-P and calcitonin gene-related peptide. These vasodilators actively influence the surrounding environment through the introduction of circulatory materials (e.g. blood, various inflammatory cells), and eventually lead to the formation of edema.[84,85,95,105] All of these factors have a sensitizing effect on nociceptors, causing a decrease in the excitatory threshold to mechanical stimuli.[85] The decrease in threshold allows the nerve to become increasingly sensitive to stimuli that are normally classified as non-noxious in nature. Prolonged activation of nociceptors, and nociceptive input however, can eventually lead to neuroplastic changes in the peripheral and central nervous systems promoting the development of various chronic pain syndromes.[84,89,94,95]

The unrestricted production of neurotrophic growth factors following the sensitization of afferent fibers, can eventually lead to collateral sprouting of the afferents in the periphery, as well as fibers within the lamina of the spinal cord.[85,89,106] Sprouting of afferents amplifies their input to various pathways within the spinal cord, potentially effecting sympathetic reflex pathways and peripheral skeletal muscle spasticity.[89,107] A potent neurotrophic growth factor, termed Nerve Growth Factor (NGF), is classified as a neuronal sensitizing agent.[85,93] NGF is released by the muscle during injury, and when uncontrolled, can lead to debilitating chronic pain syndromes.[85,89,106] A previous study showed an increase in free nerve ending fiber density in the perimysium following persistent inflammation in skeletal muscle in just over one week.[93] The mechanism proposed suggested NGF as the contributing factor to the increase in substance-P production in the dorsal root ganglion.[93] This illustrates the rapidity with which the

peripheral nervous system can become 'efficient' at pain transmission, and illustrates the importance of timely modulation of the early immune response.

3.4 The Physiological Effects of Mechanotransduction

The cytotoxic environment created via tissue damage and the immune response propagates, inducing hypoxia. The less than favorable environment that is created includes not only the musculature, but the vascular and nervous tissues as well. Furthermore, afferent nerve endings in the local area are similarly sensitive to the cytotoxic build-up, which promotes depolarization of afferent nerves and signal propagation associated with pain. Although, nerve and muscle are anatomically separate structures, they should be regarded equally when developing treatment strategies, rather than thought of independently. Muscle and nerve are not only dependent upon each other for processes such as growth, development, and maintenance, but are both mechanosensitive structures that respond to a variety of applied mechanical stimuli. Understanding the properties of mechanotransduction will help clinicians better utilize manual modalities like massage.

Mechanotransduction is defined as the transformation of a mechanical stimulus into a chemical signal, or the resulting cellular signaling cascade following an external mechanical deformation of tissue.[15] Muscle is an extremely elegant structure consisting of a very complex and intricate cellular matrix, referred to as the cytoskeleton. This structure in addition to the fibers themselves, are theorized to be sensitive to mechanical changes or perturbations. The cytoskeleton consists of numerous mechanosensitive structures such as stretch activated ion channels and focal adhesion complexes. Activation of these structures can cause depolarization, and change the sensitivity of surface receptors to their substrates, as well as serve as a major source of signal transduction within and between cells. This mechanotransduction can ultimately lead to the transmission of signals throughout the cell resulting in the alteration of protein expression.[15] For example, muscle responds specifically to overload by adding sarcomeres in parallel (hypertrophy) which increases the cross-sectional area, and also through the addition of sarcomeres in series when longitudinal stretch is applied.[15-18]

These studies show that various loads applied to muscle tissue have the ability to trigger distinct signaling cascades leading to adaptive cellular responses.

Recent evidence suggests that there is a disparity of responses within and between cells to a given mechanical stimulus.[18] The same mechanical signal may recruit a particular immune cell, but not control the function of that same cell. For instance, passive stretch is an established stimulus that recruits neutrophils, but does not necessarily cause a respiratory burst. [7] This demonstrates that neutrophil infiltration does not always lead to tissue damage associated with secondary injury.[7]

Comprehensive investigations [15,17,18] have demonstrated that the response of a muscle to perturbation depends upon the type of mechanical stimulus applied, illustrating that these signaling cascades are not random. For example, experiments using muscle cells in culture reveal that intracellular signaling in response to uniaxial (one direction) or multiaxial (multiple directions) stretch, is strain specific.[15] This observation is noteworthy from a clinical and translational perspective, because muscle is isovolumetric and sensitive to distinct perturbations; negative strains induced by axial compression of muscle arguably result in a compensatory positive longitudinal strain in other regions of the tissue. In fact, recent work [91,92] reveals muscle is more stiff when load is applied at an angle (more acute to the fiber orientation), due to the lateral movement of fluid against the plane of fiber orientation, altering shear stress. This illustrates the complexity of the tissue response (e.g. sarcolemma deformation, protein distortions, fluid flow) during tissue manipulation. With over 75 different methods associated with massage alone [23], the type of manipulation applied should be carefully considered based on the desired outcome. The use of massage versus joint mobilization will ultimately affect the response of the local tissue. The addition of a variety of techniques, adds not only to the intricacy of the effect, but the actual application as well. The importance of the location, orientation, and application of load, controlled and administered by the clinician, all become critical points to be emphasized.

Recent work [90] in the field of mechanotransduction has measured the rapidity of mechanical signal propagation from the plasma membrane to the nucleus, which is essential for cell communication and gene expression. Signals are transduced in skeletal muscle via direct connections between the cell membrane and nucleus at a rate that is six orders of magnitude greater than traditional ligand-receptor rates, ~5 μsec and 5 sec respectively.[90] Mechanotransduction exists at the sarcomere level as well. Both the Z-disk and M-band have been regarded as active signal transducers, as they transform positive and negative mechanical strain into biochemical responses for protein expression/degradation.[108] Furthermore, the giant protein titin acts as a passive tension sensor within the M-line.[108] In response to mechanical stress, a conformational change in this region of the protein exposes ATP binding sites, promoting activation.[108] This process has been linked with downstream phosphorylation and activation of various proteins associated with apoptosis (programmed cell death), autophagy (cell survival), and hypertrophic signaling (cell growth).[108]

3.5 The Immunomodulatory Effects of Massage

To discern the biochemical and cellular changes occurring with massage, our laboratory has developed a device that serves as a massage-mimetic, which allows for tuneable and highly reproducible application of force. Under these controlled conditions, a dramatic influence of massage on skeletal muscle inflammation and function was observed.[19] Application of a 30-minute bout of massage-mimetic to an eccentrically damaged rabbit tibialis anterior muscle once a day, over four consecutive days, reduced the amount of cellular infiltration and tissue necrosis compared to a non-massaged eccentrically exercised muscle.[19] Treated muscles not only recovered mechanical function at a faster rate when compared to exercised, non-massaged muscles, but histologically the massaged muscle tissue more closely resembled that of non-exercised healthy control muscles.[19] Massaged muscles exhibited little cellular infiltration and regular intracellular spacing. This was the first study to show that massage effectively reduced cellular infiltration as well as subsequent inflammation and edema, thereby facilitating recovery of function.[19] These findings, in conjunction with our ongoing work on massage and inflammation, lead us to propose massage as an immunomodulatory therapeutic modality.

The inflammatory response to damaging eccentric exercise commences immediately after the activation of muscle, and continues long after exercise ceases.[75] Damage has been related to the intensity and duration of the exercise, and is likely additive over time as cells repeatedly transduce mechanical signals to chemical responses.[41] Interestingly, the timing of the massage application with respect to exercise cessation appears to influence its immunomodulatory efficacy. Recent pilot work in our laboratory has shown that the longer the delay in massage application, the less effective it becomes for reducing secondary hypoxic injury. This finding is especially important for clinicians when developing acute treatment plans.

Recently, we have applied massage to healthy undamaged muscle to investigate its action without the confounding elements of exercise-induced damage. We showed that the magnitude of applied load has an effect on resident (M_2) and non-resident (M_1) macrophage numbers in the muscle. There also appears to be an optimal load which increases M_2 macrophage numbers in healthy skeletal muscle, suggesting a better environment for repair and regeneration [109].

Our ongoing hypothesis is that application of mechanical compressive loading (a massage-mimetic) is a potent immunomodulator following damaging exercise. Moreover, a specific combination of timing, force, and technique exists in which an optimal inflammatory environment is created that promotes tissue repair. This model of massage application and tissue response is based on our current findings and ongoing research. We propose that the magnitude of a single bout of massage modulates the levels or density, of three physiological factors: M_1 and M_2 macrophages, and afferent nerve fibers. We have not included neutrophils, as we have demonstrated previously that massage application immediately following eccentric exercise limited damage through the attenuation of secondary injury (due to respiratory burst) and limiting edema. Therefore, we are most interested in macrophage recruitment, potential phenotype transition, pain modulation, and tissue repair.

In our working model, the optimal range for massage application in the event of an injury (such as a moderate to mild contusion or tear) appears to be a low to moderate magnitude of load sufficient to influence M_1 and M_2 macrophages in a beneficial manner. These loads have the potential to modulate, elevate, or promote early activation of M_2 macrophages, suggesting an M_1 transition into repair and regeneration. We suggest that massage promotes a restorative environment, minimizes respiratory burst and pro-inflammatory cytokine release from M_1 macrophages, and limits the amount of cytotoxic chemicals in the surrounding area. Potential for afferent nerve sensitization is reduced, leading to an attenuation of nerve fiber density adaptations in the periphery, thereby preventing plastic changes at the spinal cord level.

In the event of a significant, very destructive, untreated inflammatory response, M_1 macrophages appear to remain elevated past the 48-hour peak when they typically begin to decline. An example of this type of an injury would be crushing injury, or a grade three tear in the musculature. The combination of highly elevated M_1 and M_2 macrophages indicates an extreme inflammatory condition. Increased density of macrophages in the area suggests a previous and potentially ongoing neutrophil infiltration resulting in the excessive breakdown of local tissue, promoting further pro-inflammatory cytokine signaling. Membrane lesions due to secondary injury results in an increased level of neural sensitization agents, such as NGF which propagates the action of substance-P, and increases afferent nerve fiber density. One-week post injury, changes in afferent density have the potential to cause detrimental plastic changes (collateral sprouting) in the dorsal horn of the spinal cord.

Currently, one contraindication for massage includes acute muscle injuries. However, based on our current understanding of the inflammatory response and secondary injury in skeletal muscle, we have proposed that massage can be beneficial when applied immediately post-injury.[19,97] To this end, we have been systematically studying the effects of massage application immediately following damaging eccentric exercise. Due to inherent biologic variability, we normalize muscle damage using exogenous supramaximal stimulation during eccentric exercise.[48,97,110-112] Although this represents a

non-physiological force production, it does result in a reproducible and controlled degree of muscular damage for our study of massage efficacy.[113] Subsequent application of our massage-mimetic are calculated based on allometric scaling laws and using a ratio of species-specific muscle mass and lumbar vertebrae cross sectional area.[19] Currently, we propose the translational capacity for effective loads in our laboratory could be similarly scaled for application to humans. As we reported, muscle damage responded very well to an immediate post-exercise 30-minute bout of massage, not only reducing the inflammatory response, but also accelerating functional recovery. At this time, we consider all aspects of massage application as important variables for optimal cellular response, including massage technique, type, magnitude, timing and duration of applied load, and even the nature of the injury.

One explanation behind the physiological benefits of massage may be its influence on apoptotic signaling. Apoptotic signaling of neutrophils has shown to influence a phenotype change in the macrophage population. M_1 macrophages, being phagocytic in nature, seek and engulf apoptotic cells and lysed fragments. If an M_1 macrophage engulfs an apoptotic cell, rather than its lysed parts, this can influence a phenotype change in which the M_1 macrophage transitions to an M_2 macrophage, secreting anti-inflammatory products.[1,83] Macrophages that engulf apoptotic neutrophils prevent the release of neutrophil cytotoxic chemicals, ceasing respiratory burst, and increasing the secretion of transforming growth factor-beta and IL-4.[1,83] The release of transforming growth factor-β and IL-4, result in the decrease in the release of pro-inflammatory cytokines (e.g. tumor necrosis factor-alpha and interleukin-6) promotes a transition to the Th_2 cytokine pathway [1,83] (**Figure 3.1**). The anti-inflammatory nature of the Th_2 pathway promotes the repair and regenerative process. [83,90,100]

Using massage to influence phenotype change prompting the transition into the repair and regeneration phase may play an important role in the physiological benefits of massage. Preventing the exacerbation of a toxic environment through the attenuation of neutrophil recruitment, respiratory burst, or through the phagocytosis of apoptotic neutrophils, would also greatly decrease endogenous chemical availability, and the potential of nerve

sensitization. In doing so, massage may have the ability to prevent transient and more detrimental plastic changes in afferent nerve density in the periphery, as well as the spinal cord. Attenuating the inflammatory and subsequent nervous response, may allow clinicians to treat, manage, and prevent acute and chronic pain syndromes with massage, as well as inflammatory related diseases, absent of pharmaceutical intervention.

3.6 Conclusion

Currently, massage therapy is tied to numerous indications and contraindications, with a lack of rigorous scientific evidence to reinforce present guidelines. Many critics of CAM/CIM therapy disregard its proposed effects due to the lack of randomized control trials.[114] Recent investigations have demonstrated that there is a clear physiological response to the application of massage.[19,97,98,109] Further mechanistic investigations of massage are critical to establish a better understanding of its beneficial immunomodulatory effects. Future studies should focus on the temporal nature of the various inflammatory cell populations, while attempting to limit the confounding effects of multiple bouts of massage. Additionally, study designs should attempt to closely mimic the clinical setting, and contribution to the establishment of goals and appropriate parameters for massage application are encouraged. Special attention should be given to the technique of application, as distinct cell signaling pathways may be activated with different massage strokes. Just as structure dictates function, mechanism should dictate treatment. Identifying specific signaling pathways affected by massage will provide insight into the proper clinical application through the creation of an advantageous inflammatory environment to promote repair.

CHAPTER FOUR: THE IMMUNOMODULATORY EFFECTS OF MASSAGE ON NON-PERTURBED SKELETAL MUSCLE IN RATS

4.1 Introduction

Massage is defined as a "mechanical manipulation of body tissues with rhythmical pressure and stroking for the purpose of promoting health and well-being" [115]. According to the American Massage Therapy Association the benefits of massage therapy include alleviation of pain, tension headaches, and depression, while promoting sleep, and improved quality of life [116]. Although it has recently gained a considerable presence in the health care system [25,34], the evidence in support of massage as a beneficial clinical modality remains mostly anecdotal. Most reports on the effects of massage have focused on the recovery of muscle function after exercise. Our animal models of massage indicate that massage limits damage and enables the repair and regenerative process [117]. However, a comprehensive literature review indicated that scientific evidence relating massage application to post-exercise muscle recovery and function is conflicting [21]. Moreover, mechanisms underlying the damage-reducing effects of massage are just starting to be investigated and some studies are focusing on the immune system. Increases in the number of natural killer cells [118-120], and lymphocytes [118-121], as well as their cytotoxic capacity [119,122] have been observed with massage in immune-compromised individuals (AIDS/HIV) [119,120], subjects with cancer [118], pre-term infants [122], and healthy individuals [121,123]. Increases in cell number occur after one day [123] to several weeks of intervention. These studies provide important evidence for the influence of massage on the immune response, however they also greatly underscore the methodological limitations for massage studies involving human subjects, such as application technique, the amount of load applied, and frequency and duration of sessions.

The fabrication and utilization of a novel massage device which applies pre-determined loads to muscle has allowed for a more controlled exploration of the mechanical effects of massage in skeletal muscle [19]. Cyclic compressive loading, a massage mimetic, enhanced functional recovery of force production when immediately applied after a bout of eccentric exercise to tibialis anterior muscle in a rabbit model [19], and also showed

recovery of the muscle's mechanical properties such as stress relaxation and creep [26]. Cellular mechanisms that potentially contribute to the recovery of function are starting to be investigated. It was noted that leukocyte infiltration was decreased in exercised muscle after massage in rabbits [19] and biopsies taken from exercised and subsequently massaged quadriceps muscles of human volunteers, showed an attenuation of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-a), and interleukin-6 (IL-6) [98]. A recent investigation demonstrated a reduction in inflammatory cells in muscle that was immediately subjected to massage after exercise, compared to muscles that were not massaged [20]. This immunomodulatory effect was also found to be time-dependent such that the functional recovery was attenuated if the application of massage was delayed after exercise [20]. Collectively, these studies indicate that massage modulates the inflammatory response in skeletal muscle following exercise, and consequently influences secondary muscle damage, which is usually associated with inflammation [124].

Although it is becoming clear that the immune system is involved in response to massage after a damaging event, it remains to be determined whether non-injured muscle responds in similar fashion and if the immune system is involved when previous muscle damage is not induced. Additionally, differential responses provoked by variable loads applied to unperturbed muscle, have yet to be evaluated. Therefore, the purpose of this study was to investigate the immunomodulatory responses to massage of non-damaged, unperturbed muscle in relation to magnitude of applied load. We hypothesize that massage induces a beneficial inflammatory environment, which is load dependent. Utilizing our custom fabricated cyclic compressive loading device modified from the previously described device [19,20,26,97,124], we will determine the effect of massage on rat tibialis anterior muscle in response to different load magnitudes.

4.2 Methods

Twenty-four male Wistar rats (200g, Harlan Laboratories, Indianapolis, IN) were used in this study. Rats were housed in cages within the animal housing facility at the University of Kentucky, with access to food and water ad libitum. All procedures were approved by the University of Kentucky's Institutional Animal Care and Use Committee. Rats were

randomly assigned to one of four massage groups (n=6): control at 0N (C); low load at 1.4N (LL); moderate load at 4.5N (ML); and high load at 11N (HL). The HL was determined from previous work with rabbits [19], the LL is the minimal load applied by the cyclic compressive loading device (CCLD), and the ML load was determined by scaling down the optimal load determined for rabbit tibialis anterior for use in rats. All groups (excluding control group) received a bout of cyclic compressive loading (CCL) for 30-minutes (on their right limb) over four consecutive days.

4.2.1Cyclic Compressive Loading of Muscle

Rats were anesthetized using isoflurane and placed lateral recumbent on a heated sling with one limb secured to a small platform. The tibialis anterior (TA) muscle was placed facing superiorly for the application of cyclic compressive loads by a custom fabricated, cyclic compressive loading device (CCLD) adapted for rats **(Figure 4.1)** [19]. A spring loaded strut mechanism was designed to allow a cylinder to roll longitudinally over a contoured mass of tissue and displace vertically in response to the normal force exerted upwards from the tissue to the roller during an oscillating movement. Utilizing a Hookean model, the vertical (y) displacement of the roller (5) was resisted by adjusting the deformation length of two identical compressive springs (3) attached to the struts (4,6) suspending the roller's axle **(Figure 4.1).** The desired amount of preloaded force was adjusted using a rigidly fixed micrometer (1) head with a stroke length of 25mm and a resolution of 0.01mm **(Figure 4.1)**. A force transducer (2) was mounted in series between the micrometer head (1) and compression spring loading mechanism (3), which, after calibration, enabled continuous, real-time readings of the normal force applied to the roller. The output signal from the force transducer was routed through strain-gauge signal conditioning amplifier and low pass filtered at 100Hz through an integrated second order recursive Butterworth filter (Vishay 2310B, Vishay Micro-Measurements, Raleigh NC). The spring loaded roller mechanism was calibrated prior to application of CCL by displacing the roller using a DFS digital v-block mounted to a force gauge (Shimpo, Itasca, IL). Positioned directly below the mechanism the micrometer was adjusted every 0.5mm throughout its range while recording the output voltage of the transducer, and force gauge at each position.

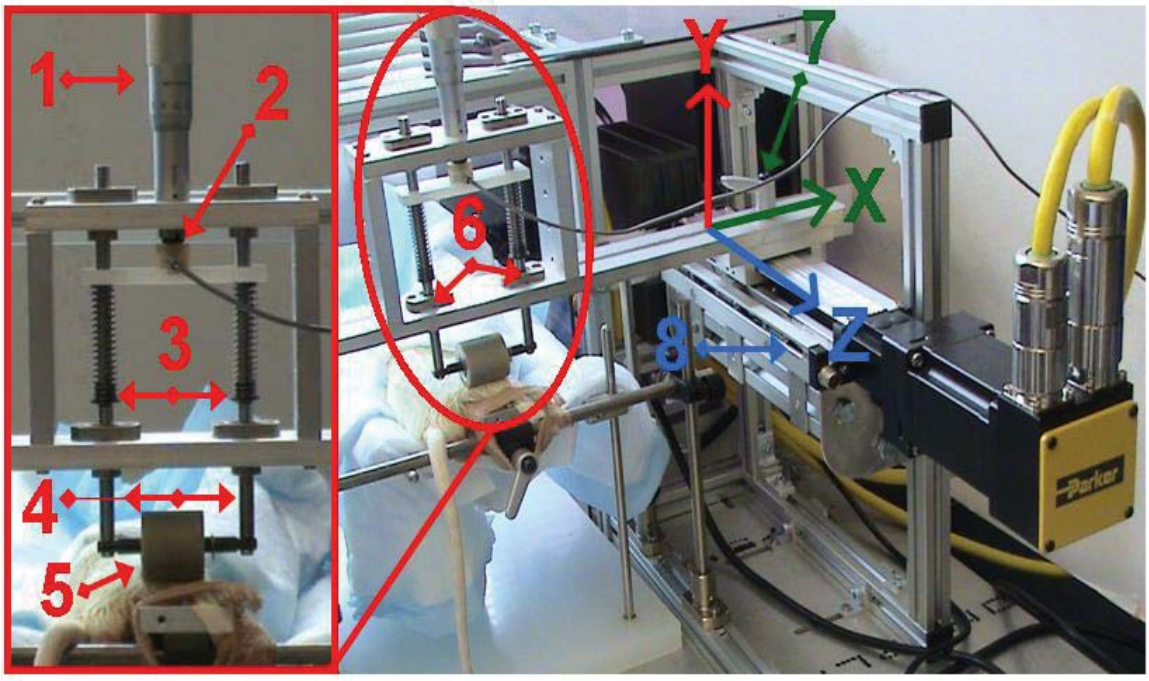


Figure 4.1: Cyclic Compressive Loading Device (CCLD)

Custom fabricated CCLD has the ability to quantify compressive loads to soft tissues during cyclic motions, from a range of 1.4N to 100N utilizing a spring-loaded roller mechanism. A vertically mounted micrometer (1) allows precise adjustment of the compressive load applied to two springs (3) arranged in parallel with a known Hooke's spring constant, to allow accurate calibration of forces applied through the 25mm roller (5) in series with a force transducer (2). Struts (4) rigidly attached to the roller's axle and distal end of compressive springs to allow vertical movement (y). The horizontal position (x) of the roller assembly was secured with clamps (7) and linear translation (z), stroke length, and frequency were controlled by a servo-motor and linear actuator (8) providing positional feedback and data in real time.

The horizontal position (x) of the roller system, and subsequently the contact point of the roller, was controlled using a linear actuator with a stroke length (z) of 120mm driven by a servomotor (8) (Parker Hannifin Corporation, Irwin, PA), controlled via Motion Planner© software, (Compumotor, Rohnert Park, CA) **(Figure 2)**. Data from the motor and force transducer were acquired with WinDaq® data acquisition (Dataq Instruments, Akron, OH) at 250 samples per second.

For CCL application, the roller was placed over the skin overlying the TA muscle, immediately proximal to the lateral malleolus of the hindlimb, and cycled 15mm proximal and distal along the length of the TA muscle at a frequency of 0.5 Hz. The contralateral left limb in all groups was not subjected to massage. The control group (0N load) was anesthetized and placed lateral recumbent on the CCLD platform for 30-minutes. Upon completion of the massage session or sham treatment the rats were returned to their cages and allowed to recover every day.

Rats were euthanized 24 hours after the fourth massage session by IP injection of Euthasol (sodium pentobarbital) (NLS Animal Health, Pittsburgh, PA). The TA muscles from both legs were dissected, sectioned into medial and lateral portions, flash frozen in liquid nitrogen and maintained at -80^{0}C for future analysis.

4.2.2 Microarray Analysis

Total RNA was isolated from the TA muscle using the ToTALLY RNA™ kit (Ambion-Applied Biosystems Foster City, CA), according to manufacturer's instructions. RNA integrity and concentration were determined using the Agilent (Agilent Bioanalyzer 2100 Palo Alto, CA). Microarray was used as a screening tool to determine changes in gene transcription with compressive load. Real time RT-PCR was then utilized to verify findings. Hypothesizing that the high load of 11N would cause a substantial inflammatory response, only Control, LL, and ML tissues were subjected to microarray and RT-PCR analysis. Samples with a RNA Integrity Number (RIN) of 8 or greater were analyzed at the Microarray Core Facility at the University of Kentucky. Three samples, one sample per chip (Affymetrix GeneChip™), were assayed from each group: Control,

LL, and ML. We were most interested in determining the differences that may exist between the LL and ML for therapeutic benefit. The microarray was performed using Command and Expression Console Software (Affymetrix). Output generated was analyzed using Partek software version 6.5 (Partek Inc.). Statistical analysis of the microarray was performed using Robust Multichip Average (RMA) for chip normalization. Following normalization a one-way ANOVA ($p<0.01$ significance) was used to compare treatment groups at the gene level. Genes which were found to be differentially expressed were analyzed for gene ontology using Database for Annotation, Visualization, and Integrated Discovery (DAVID). Functional Annotated Clusters meeting an acceptable enrichment score of ≥ 1.3 (minus log scale, equivalent or less than significance of 0.05) [125] under medium stringency (15 total) were included in the overall analysis. Patterns of gene expression were determined for differentially expressed genes. Individual genes were selected for verification via RT-PCR based on a 1.8 fold change cut-off and mean overall abundance.

4.2.3 RT-PCR

RNA was isolated using the RNeasy Fibrous Tissue Mini Kit (*Qiagen Valencia, CA*) according to the manufactures instructions. RNA integrity and concentration were determined using the Agilent (Agilent Bioanalyzer 2100 Palo Alto, CA) in addition to Nano Drop (Thermo Fisher Scientific, West Palm Beach, FL). cDNA was obtained from 400ng RNA with RIN of 8 or higher using ReadyScript™ cDNA Synthesis Mix according to manufacturer's instructions (Sigma-Aldrich, St. Louis, MO). cDNA concentration was measured using a Nano Drop (Thermo Fisher Scientific) and samples were diluted to 50ng/µl per well for RT-PCR analysis. Primers were designed using TaqMan® Probe and Primers from Applied Biosystems (Foster City, CA), (Rn primer numbers: CXCR5: Rn02132880_s1, CD74: Rn00565062_m1, CCR2: Rn01637698_s1, LYZ2: Rn00562794_m1, LILRB4: Rn01399943_m1). PCR reactions were assembled using protocols from Applied Biosciences utilizing TaqMan® Gene Expression Master Mix and protocol (Applied Biosystems). Reactions were performed using the ABI Prism 7700 Sequence Detection System (Applied Biosystems) using standard cycling conditions.

4.2.4 Hematoxylin and Eosin Staining

TA muscle cross sections were cut at 8μm; every fifth section was fixed using methanol (100%) and reacted for standard hematoxylin and eosin staining. A total of four sections per muscle were analyzed.

4.2.5 Immunohistochemistry

Neutrophil and Macrophage Quantification: TA muscle cross-sections were cut as above and fixed in ice-cold acetone (100%). Sections were blocked in 3% H_2O_2 in PBS, followed by normal horse serum (ImmPRESS-Vector Laboratories Burlingame, CA). Primary antibodies were applied and incubated overnight at 4°C at 1:100 dilution for (1) Neutrophils: Mouse anti-Rat CD43; (2) M1 macrophages: $ED1^+$ Mouse anti-Rat CD68; and (3) M2 macrophages: $ED2^+$ Mouse anti-Rat CD163 (Serotec Raleigh, NC). ImmPRESS anti-Mouse IgG (*Vector Laboratories*) secondary antibody with fluorescein (for M1 and M2) or cyanine-3 (Cy3 for neutrophils) was applied in amplification buffer (Tyramide Signal Amplification (TSA) *Perkin Elmer Waltham, Mass.).* 4'6-diamidino-2-phenoylindole (DAPI) was applied at 0.01μM to visualize nuclei. All histology images were obtained using a Zeiss Axio Imager M1 microscope (*Carl Zeiss Microimaging GmbH Göttingen, Germany)*

4.2.6 Stereological Point Counting

Total cellular abundance was measured using a random stereological point counting technique. Using a Zeiss Axio Imager M1 microscope at 200x magnification, one randomly selected field from each of the four sections per muscle was photographed and used for cell counting. Cells in the interstitial space outside muscle fibers were counted as total cellular abundance on the H&E slides. For determination of ED1 and ED2 cellular abundance, we counted cells that were positive for primary antibody and also reacted with DAPI. Cell counts from each of the four sections per muscle were averaged, and expressed as cells per field of $0.15mm^2$.

4.2.7 Statistical analyses

Microarray statistical analyses were performed as described above. All remaining statistical analyses were performed using IBM SPSS 18.0 (SPSS, Chicago, IL) and SigmaPlot (Systat Software Inc. San Jose, CA). For all parameters measured, mean and standard error are reported. Levene's statistic for homogeneity of variance was violated across groups when comparing gene abundance following RT-PCR. Therefore, five one-way Welch's ANOVAs were performed with Games-Howell post-hoc tests to determine significant differences amongst groups. Student's paired t-tests were used to compare differences between designated massaged hindlimbs and the contralateral control hindlimbs for each condition (C, LL, ML, HL). One-way ANOVAs with Holm-Sidak post hoc analysis were used to compare significant differences between groups. Biological data violating normality were log10 transformed prior to one-way ANOVA analysis. Simple linear regressions were performed to assess the relationships between magnitude of load and cellular abundance for both the massaged and non-massaged limbs (H&E, M1, M2 macrophages, and neutrophils). Statistical significance was assumed at $p \leq 0.05$.

4.3 Results

4.3.1 Microarray and Gene Ontology

Microarray analysis was performed to identify differences in gene expression profiles between groups with distinct magnitudes of cyclic compression loading. Results indicated that 534 genes were differentially expressed between the three groups. Functional annotated clustering resulted in 107 clusters, 15 of which met the cut-off criteria of an acceptable enrichment score of ≥ 1.3 [125]. These clusters were combined into five categories based on associated functions to better represent the systems most affected by massage treatment **(Figure 4.2)**. Interestingly, 47% of the functional clusters were associated with 'immunity' or the 'immune response', indicating that massage treatment is associated with changes in immune function. Other clusters that were highly represented consisted of genes that have functions involving vacuoles/ lysosomes, hormone responses, respiratory transport/mitochondria, and ATPase activity **(Figure 4.2)**.

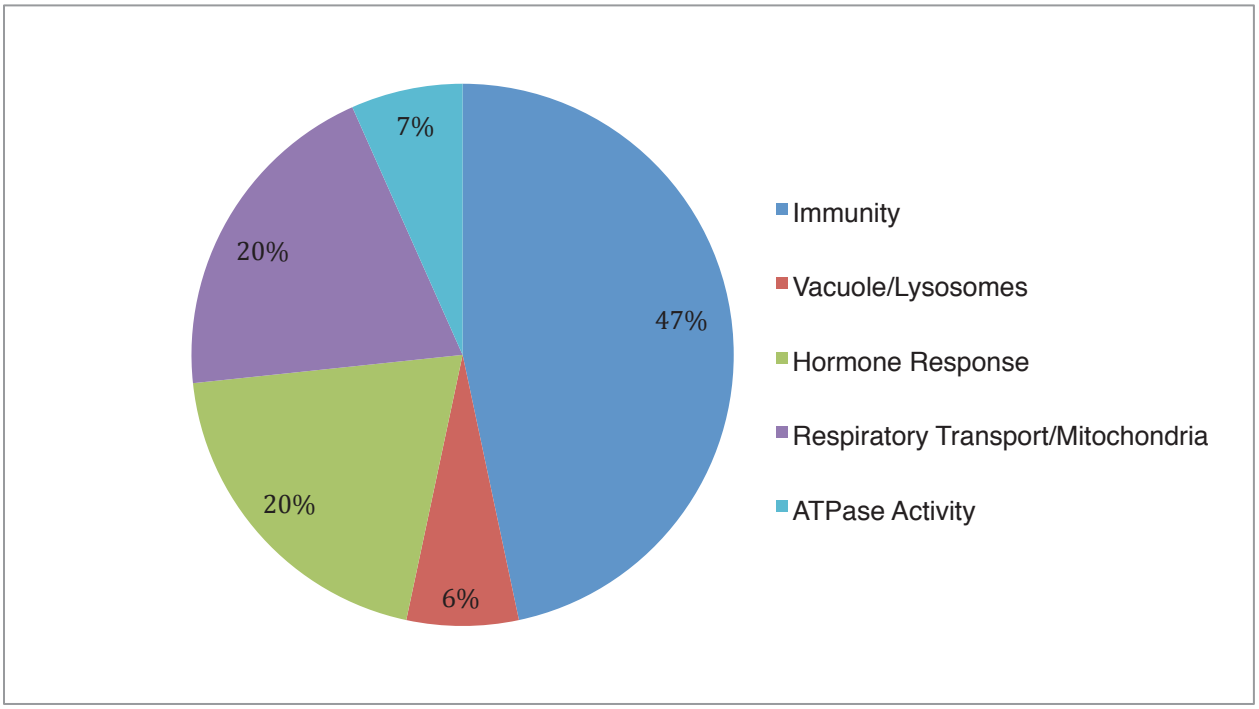


Figure 4.2: Immune related genes are abundantly changed in response to massage

Percentage of Associated Clusters. Representation of the systems most affected by massage intervention after combining 15 significant gene clusters with associated systems meeting an enrichment score of ≥1.3 from DAVID.

Expression pattern analysis was performed using significant genes meeting a minimum 1.2 fold change in either the LL or ML condition, and were mapped for mechanical load dependent changes. A total of 13 genes were down-regulated from Control to LL and remained relatively constant from LL to ML **(Figure 4.3A)**; 60 genes were slightly up-regulated or remained constant from Control to LL and down-regulated from LL to ML **(Figure 4.3B)**; 72 genes were slightly up-regulated or remained constant from Control to LL and up-regulated from LL to ML **(Figure 4.3C)**; and the majority of genes within the 1.2 cut off (74) were down-regulated from Control to LL and either remained constant or were up-regulated from LL to ML (Figure 3D). A majority of significant gene expression changes take place under the ML conditions **(Figures 4.3B, C, & D)**.

4.3.2 RT-PCR Quantification

Five genes were selected to corroborate the array results: Chemokine (C-C motif) receptor-2 (CCR2), Leukocyte immunoglobulin-like receptor (subfamily B, member 4) (LILRB4), Cd74 molecule major histocompatibility complex (Class II) (CD74), Lysozyme 2 (LYZ2), and Chemokine (C-X-C motif) receptor-5 (CXCR5). These genes showed at least a 1.8 fold increase or decrease in expression between any of the three conditions, and were of interest because of their association with the immune response **(Figure 4.4)**. No change in CCR2 gene expression was detected from Control to LL in either microarray **(Figure 4.4A)** or RT-PCR **(Figure 4.4B)**. However, CCR2 gene expression was elevated in ML compared to control and LL using both techniques **(Figure 4.4A and B)**. Gene expression pattern for LILRB4 was very similar to CCR2 such that no difference was observed between Control and LL, but an elevated level was detected in ML compared to control and LL using both microarray **(Figure 4.4C)** and RT-PCR **(Figure 4.4D)**. CD74 gene expression was also not different between control and LL, and again a higher level of CD74 gene expression was observed in ML compared to control and LL for both techniques **(Figure 4.4E and F)**. Therefore, a load dependent increase was observed for all three of these genes, indicating their involvement with massage at moderate loads. Lyz2 gene expression as measured by microarray was higher in the ML compared to Control and LL **(Figure 4.4G)**, but the RT-PCR analysis did not confirm these data even though a similar pattern was observed **(Figure 4.4H)**.

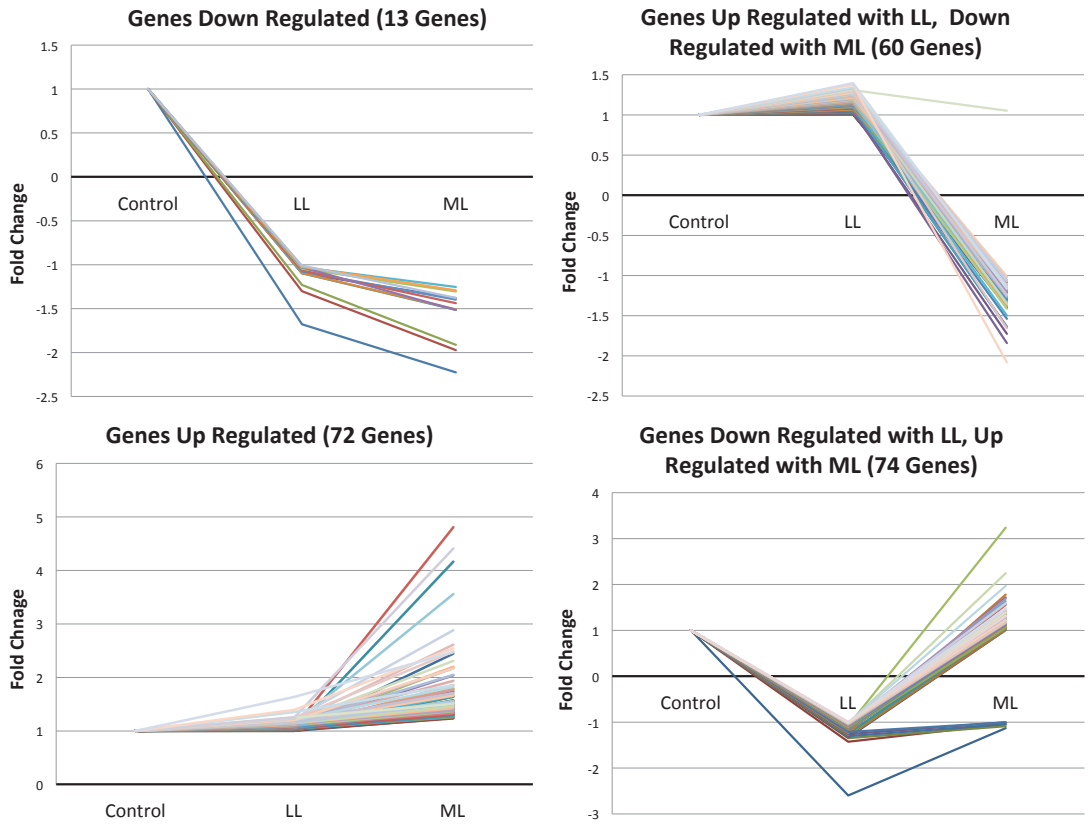


Figure 4.3 Load dependent gene expression changes

Genes meeting a minimum 1.2 fold change in either the LL or ML condition were mapped for load dependent changes. A) Set of genes down-regulated with both LL and ML; B) Genes up-regulated with LL and down-regulated with ML; C) Genes up-regulated with both LL and ML and D) Genes down-regulated with LL and up-regulated with ML. Not all genes mapped display significant patterns.

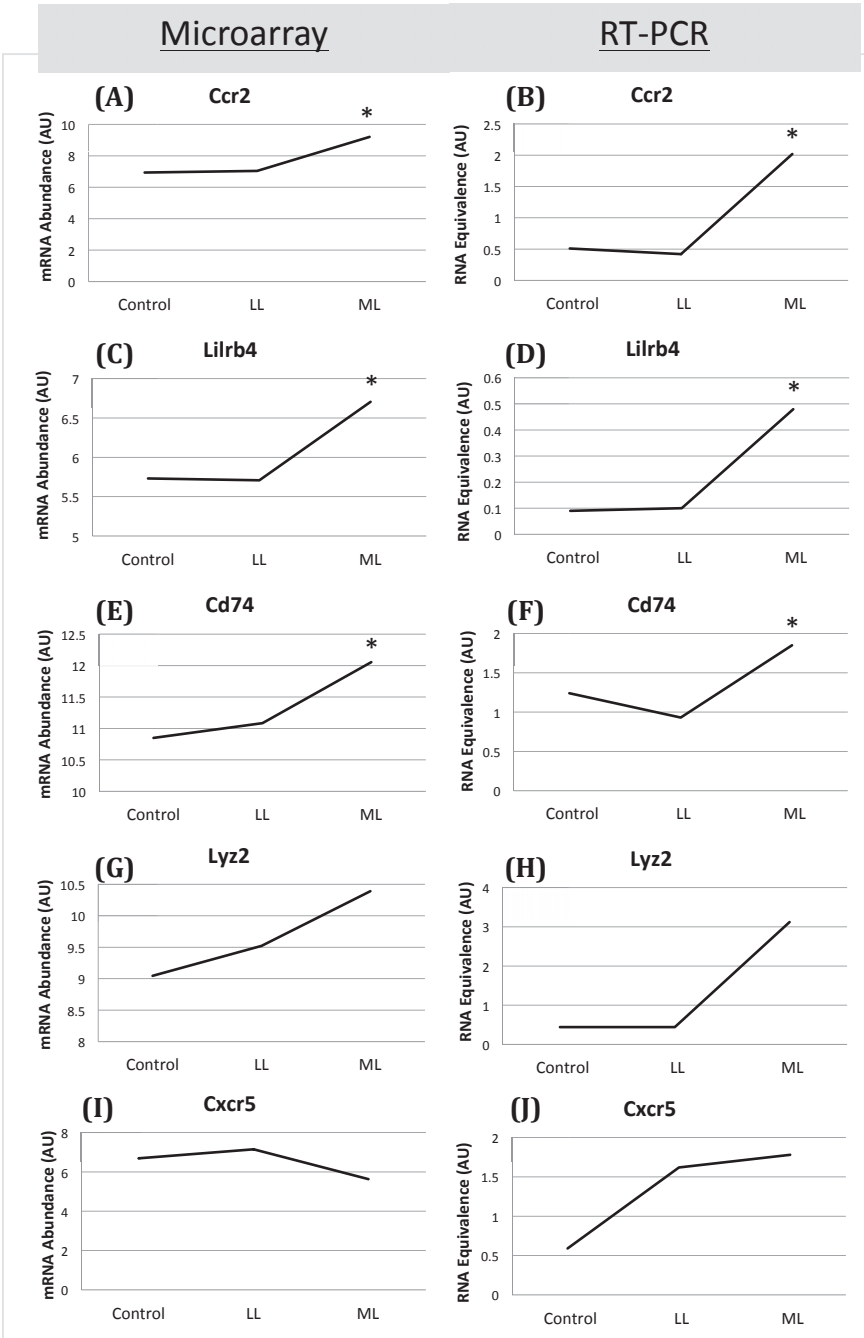

Microarray
RT-PCR
(A)
Ccr2
mRNA Abundance (AU)
(B)
Ccr2
RNA Equivalence (AU)
(C)
Lilrb4
mRNA Abundance (AU)
(D)
Lilrb4
RNA Equivalence (AU)
(E)
Cd74
mRNA Abundance (AU)
(F)
Cd74
RNA Equivalence (AU)
(G)
Lyz2
mRNA Abundance (AU)
(H)
Lyz2
RNA Equivalence (AU)
(I)
Cxcr5
mRNA Abundance (AU)
(J)
Cxcr5
RNA Equivalence (AU)
Control
LL
ML
*

Figure 4.4 (Continued). Verification of mRNA Abundance for Selected Genes

Side-by-side comparisons of gene abundance per loading condition, as measured by microarray and RT-PCR. Five genes meeting a minimum fold change of 1.8 were selected to corroborate microarray results. Chemokine (CC-motif) receptor-2 (CCR2), Leukocyte immunoglobulin-like receptor (subfamilty B, member 4) (LILRB4), Cd74 molecule major histocompatibility complex (Class II) (CD74), Lysozyme 2 (LYZ2), and Chemokine (C-X-C motif) receptor-5 (CXCR5). * Indicates significant difference from both Control and LL conditions.

CXCR5 gene expression was elevated in ML compared to control and LL when measured by microarray **(Figure 4.4I)**, but these data were not corroborated by RT-PCR analysis **(Figure 4.4J)**. In general the results from the microarray were mimicked by RT-PCR analysis and therefore immune related processes involved in the response to massage were further investigated.

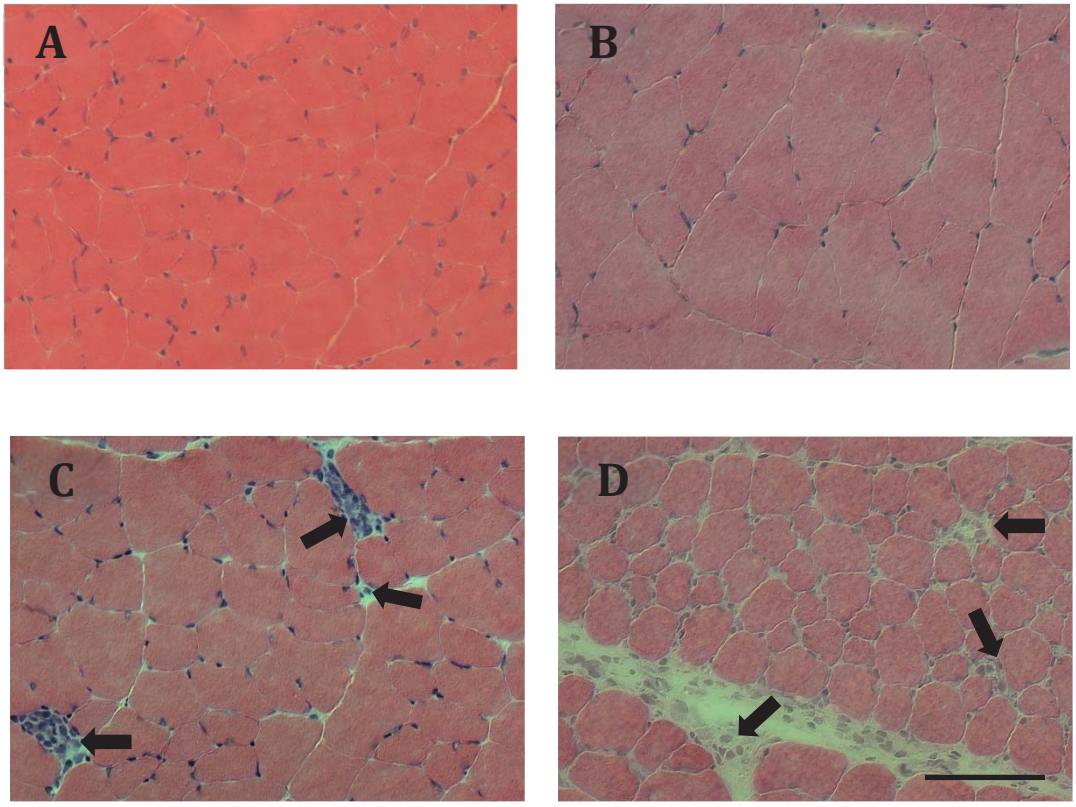
4.3.3 Histological Analysis

No gross morphological differences in cellular integrity were observed between the different load levels as seen on the H&E slides **(Figure 4.5A-D)**. The cellular immune response to massage was investigated in the TA muscle by counting the number of interstitial cells. A load-dependent increase in cellular abundance was observed in the ML and HL massaged legs **(Figure 4.5E)**. Using a simple linear regression model, the fitted data resulted in R^2=0.57, indicating the 57% of the variability in cellular infiltration could be accounted for by magnitude of the applied load **(Table 4.1)**. Surprisingly, cellular abundance observed in the contralateral non-massaged limbs was significantly higher for LL, ML, and HL, versus the reference non-massaged control animals, suggesting a systemic physiological effect of massage on cellular infiltration **(Figure 4.5E)**. This systemic 'cross-over' effect is however not load-dependent such that higher massage loads do not increase cellular abundance in the contralateral limb (p-value= 0.080).

4.3.4 Immune Cell Abundance

Key monocytes involved in the inflammatory response were analyzed. Neutrophil staining was rare in TA muscle sections of all conditions **(Figure 4.6A-D)**, no significant differences in neutrophil abundance were found in either the massage treatment, the contralateral non-massaged limb, or between limbs across all conditions **(Figure 4.6E)**.

M1 macrophage abundance was rare or absent in control conditions, but was elevated with increasing loads (Figure 4.7A-D). Following four consecutive days of CCL, muscles receiving HL had a significantly larger quantity of M1 macrophages compared to Control, LL, and ML, while ML was higher than control **(Figure 4.7E)**.


A
B
C
D

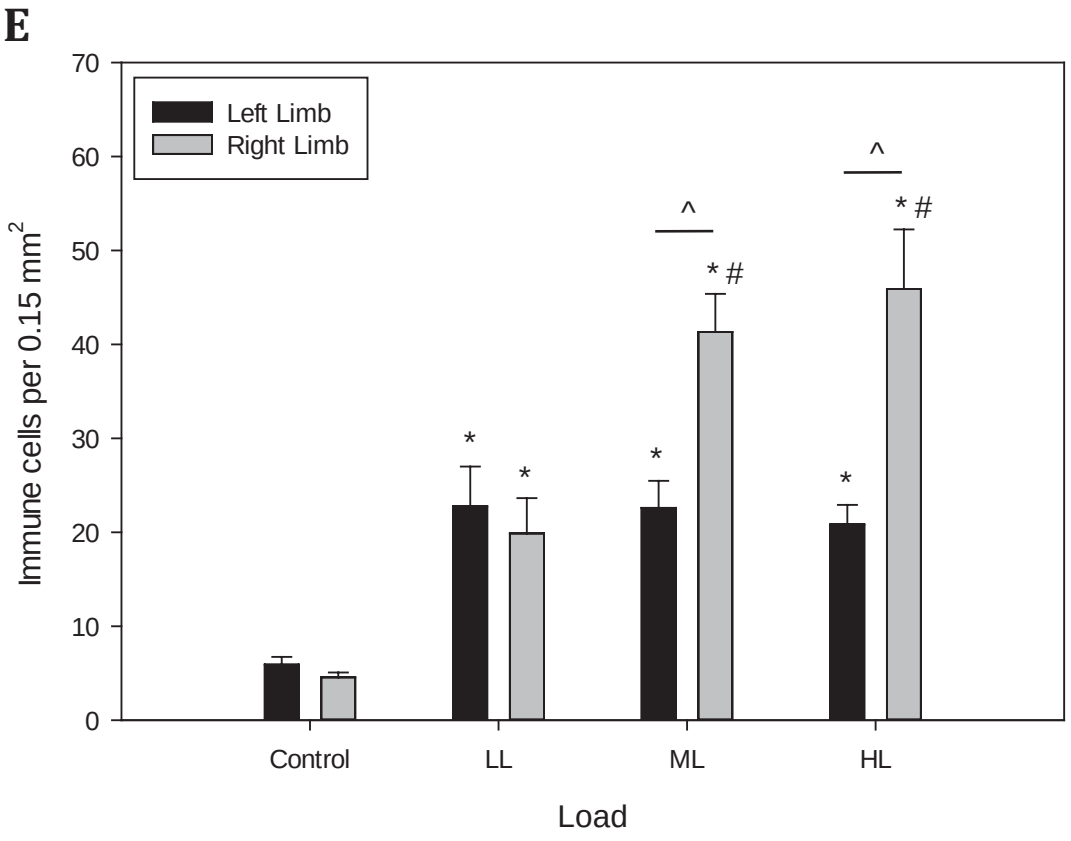
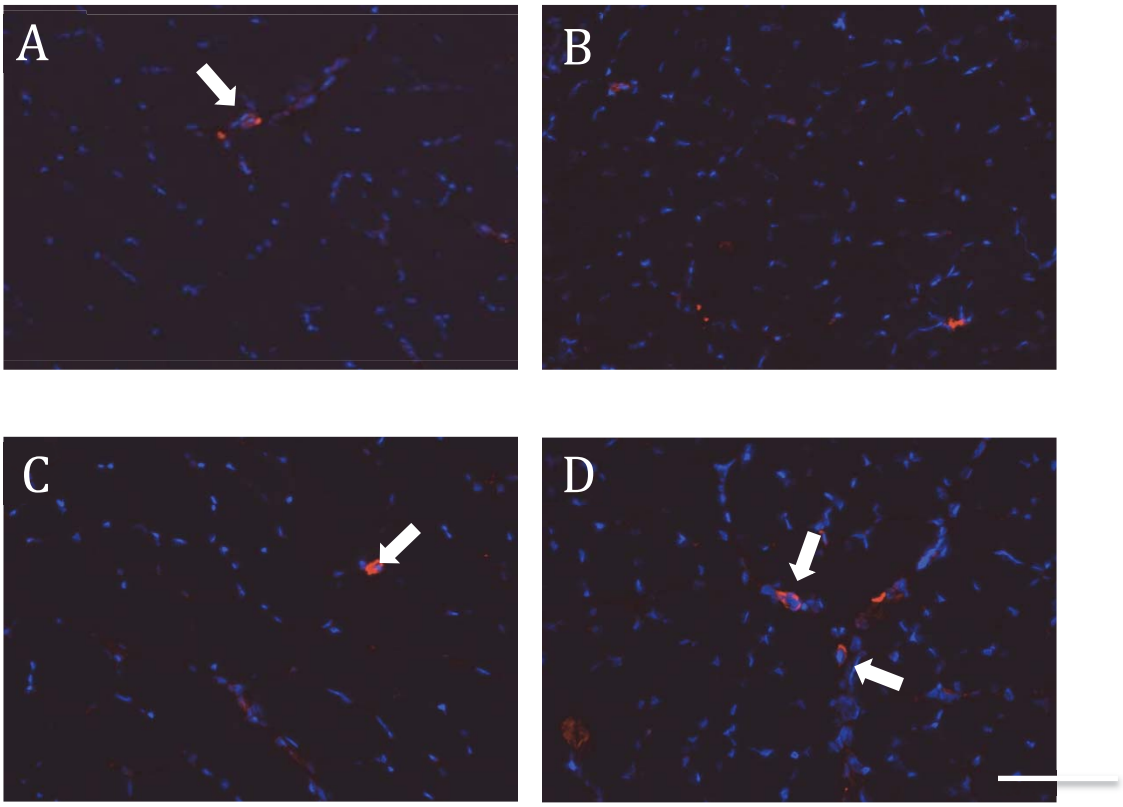

E
Left Limb
Right Limb
Immune cells per 0.15 mm2
70
60
50
40
30
20
10
0
Control
LL
ML
HL
Load
*
*
*
*
*
*
*#
*#
^
^

Figure 4.5 (Continued): Load dependent increase in cellular abundance with massage.

Representative cross sections of the right, massaged TA muscle stained for H&E of control (A), low load (B), moderate load (C) and high load (D). Bar in (D) indicates 100μm. Cell quantification across loading conditions (E) with values shown as mean ± SE. No differences were detected between limbs in the control condition therefore one bar is shown for simplification. * Significant difference from Control. # Significant difference from LL. ^ Significant difference between limbs within loading group.


A
B
C
D

E

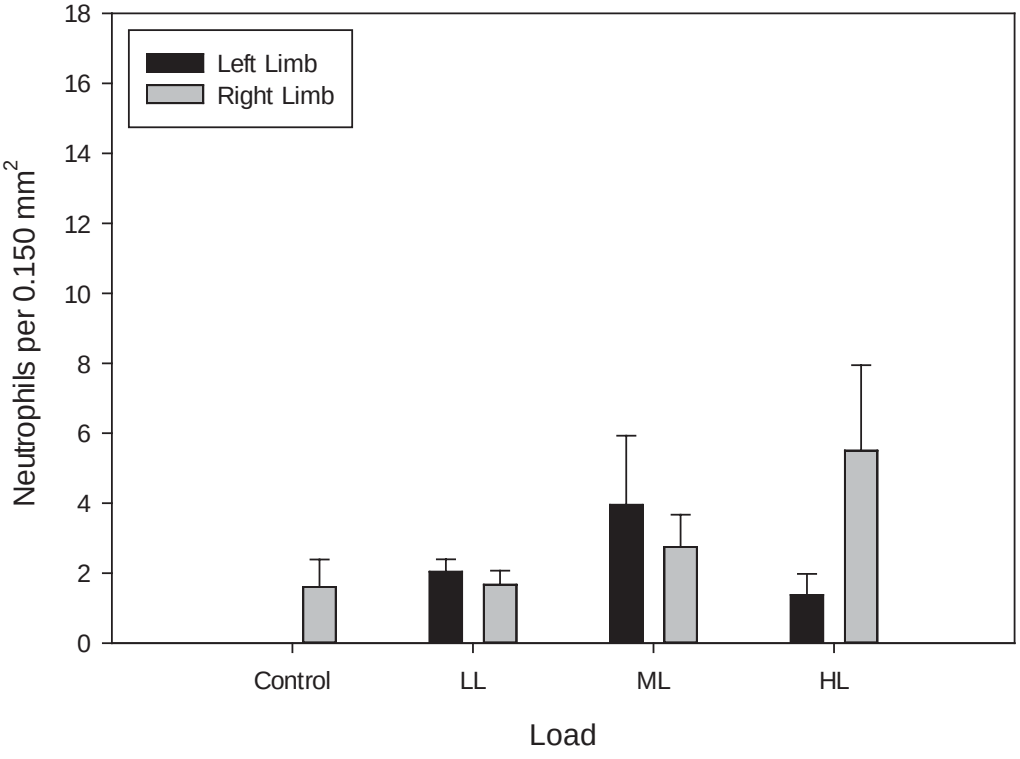
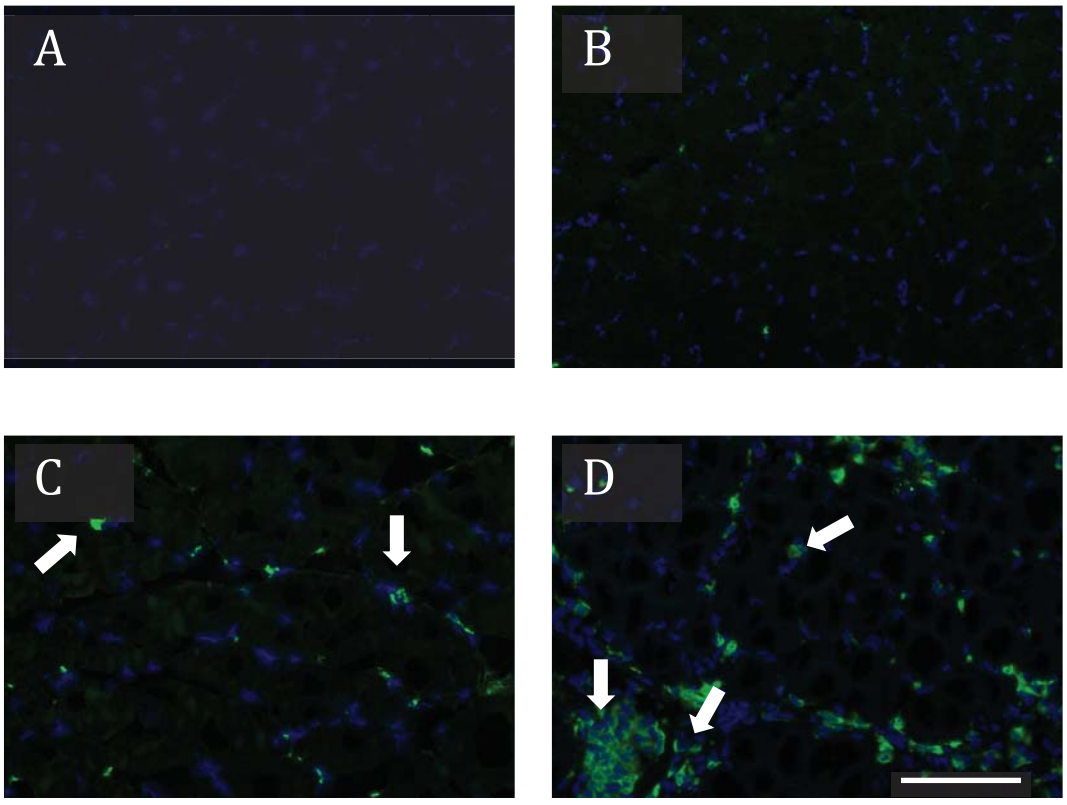

Left Limb
Right Limb
Neutrophils per 0.150 mm2
0
2
4
6
8
10
12
14
16
18
Control
LL
ML
HL
Load

Figure 4.6 (Continued): Neutrophil abundance is not different after 4 bouts of massage.

Representative cross sections of the right, massaged TA muscle of Control (A), Low Load (B), Moderate Load (C), and High Load (D) immunoreacted for CD43 as a marker for neutrophils (red) and stained for DAPI (blue). Bar in (D) indicates 100μm . Quantification of neutrophil number (E) with values as means ± SE. No differences were detected between limbs in the control condition therefore one bar is shown for simplification.

For the LL and ML no differences were detected between the massaged and the contralateral non-massaged limb **(Figure 4.7E)**. However, M1 macrophage abundance was significantly higher in the massaged limb of the HL group only **(Figure 4.7E)**, indicating that massage at a higher load induces a local pro-inflammatory response. M2 macrophage presence was detected in TA muscles across all loading groups with a higher level of staining in the HL group **(Figure 4.8A-D)**. Quantification indicated a significantly higher abundance of M2 macrophages in the HL group compared to control, LL, and ML **(Figure 4.8E)**. In addition, M2 macrophage abundance was significantly higher in the massaged limb compared to the non-massaged contralateral limb of the HL group only **(Figure 4.8E)**, indicating a pro-inflammatory immune response.

Similar to total cellular infiltrate, M1, M2, and neutrophil abundance were all found to be load dependent in the massaged limb but not within the contralateral non-massaged limb **(Table 1)**. Although the resultant R^2 value is low at 0.20, and significance was not detected when comparing within group, the regression for neutrophil abundance is significant as they do become more abundant at high loads **(Table 1)**. These correlations suggest induction of a pro-inflammatory response with the application of high load massage.


A
B
C
D

E

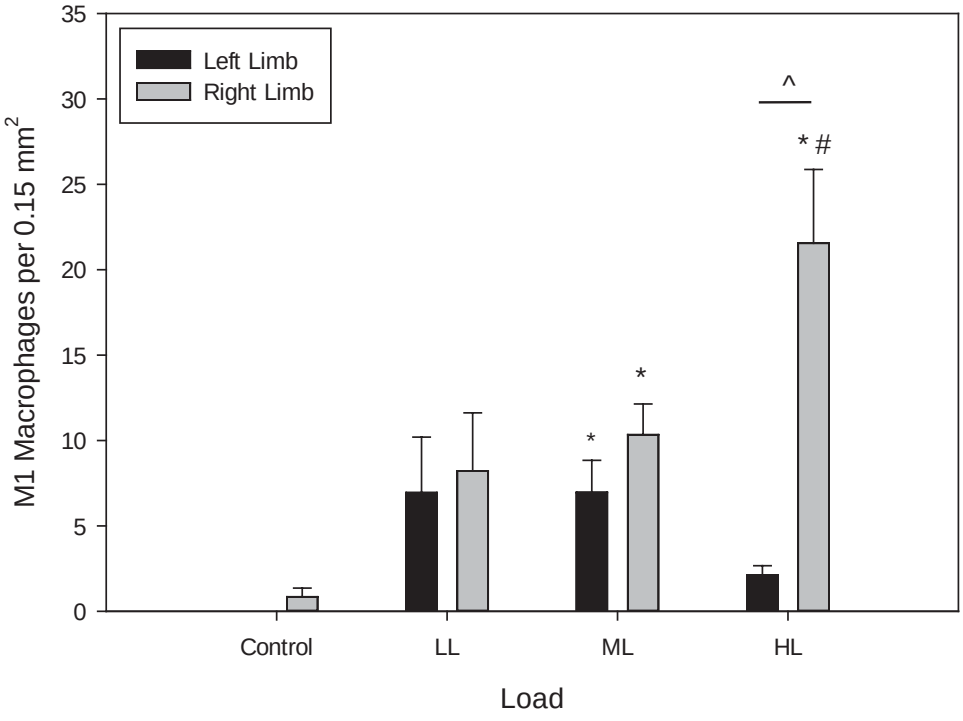
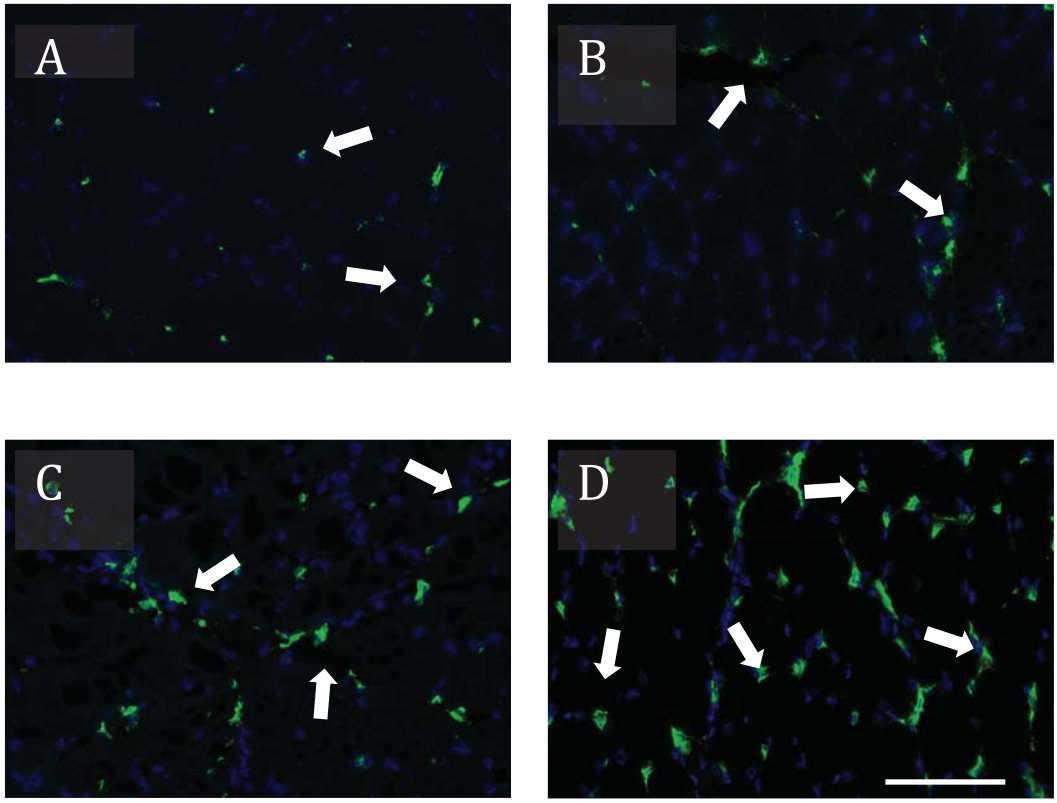

Left Limb
Right Limb
M1 Macrophages per 0.15 mm2
35
30
25
20
15
10
5
0
Control
LL
ML
HL
Load
*
*
*
*#
^

Figure 4.7 (Continued): M1 Macrophages increase in a load dependent manner

Representative cross sections of the right, massaged TA muscle of Control (A), Low Load (B), Moderate Load (C), and High Load (D) immunoreacted for CD68, a marker for M1 macrophages (green) and stained for DAPI (blue). Bar in (D) indicates 100μm. Quantification of M1 macrophage number with values as means ± SE. No differences were detected between limbs in the control condition therefore one bar is shown for simplification.* Significant difference from Control. # Significant difference from LL. ^ Significant difference between limbs within loading group.


A
B
C
D

E

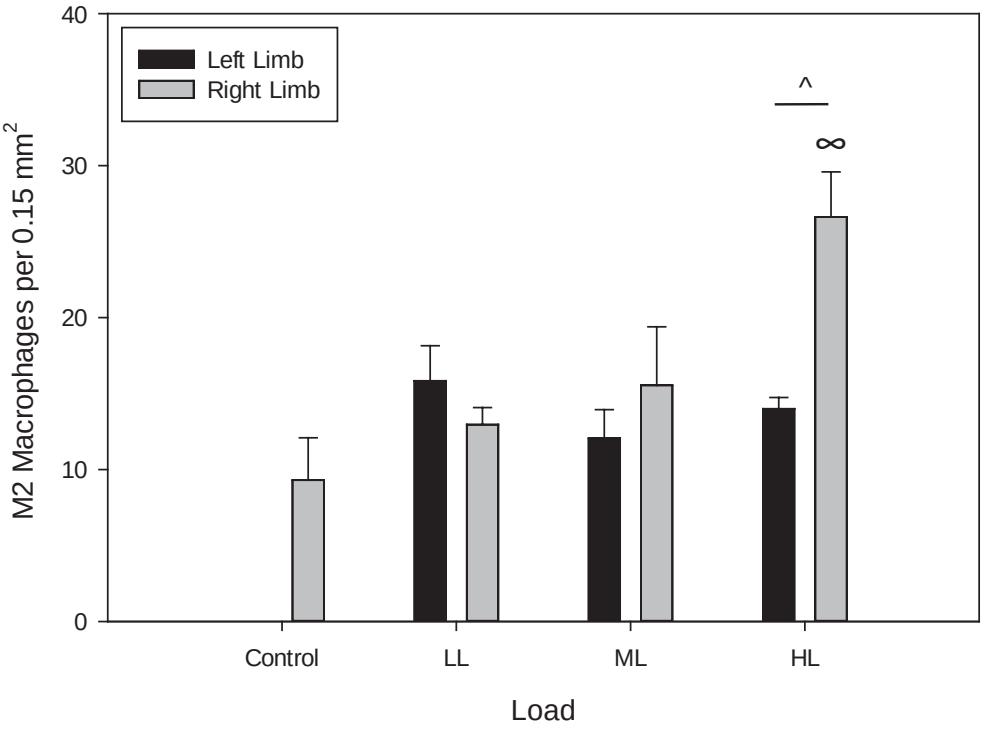

Left Limb
Right Limb
M2 Macrophages per 0.15 mm2
40
30
20
10
0
^
∞
Control
LL
ML
HL
Load

Figure 4.8 (Continued): M2 macrophages respond only to high load

Representative cross sections of the right, massaged TA muscle of Control (A), Low Load (B), Moderate Load (C), and High Load (D) immunoreacted for CD163, a marker for M2 macrophages (green) and stained for DAPI (blue). Bar in (D) indicates 100μm. Quantification of M2 macrophage number with values as means ± SE. No differences were detected between limbs in the control condition therefore one bar is shown for simplification. ∞ Significant difference from Control, LL, and ML. ^ Significant difference between limbs within loading groups.

Table 4.1: Cellular abundance is load dependent in the massaged limb and load independent in the contralateral limb

	Massaged Limb		Contralateral Non-Massaged Limb	
	R^2	p-value	R^2	p-value
Total Cells	0.57	<0.001	0.13	0.08
Neutrophils	0.20	0.030	0.00	0.760
M1 Macrophages	0.54	<0.001	0.01	0.632
M2 Macrophages	0.51	<0.001	0.02	0.509

4.4 Discussion

Beneficial physiological responses to massage have been observed in muscle, which has been previously injured [19-21,26,97,98]. However, the mechanism(s) by which massage exerts its effects, and how different loads change the response in non-damaged muscle, are currently unknown. Therefore, the aim of this investigation was to determine potential immunomodulatory targets of massage application in non-injured, unperturbed tissue relative to magnitude of applied load. Our results demonstrate that: 1) expression of genes associated with the immune response were the most affected with the application of massage; 2) various genes involved with the inflammatory response display mechanodependency, and have disparate responses to the magnitude of load applied to the muscle; 3) magnitude of applied load significantly influences the abundance of immune cells in the muscle; 4) a significant increase in both M1 and M2 macrophage abundance is observed in muscle subjected to higher loads of massage, signifying an inflammatory response; and 5) massage has a systemic effect, increasing abundance of immune cells in the contralateral non-massaged limb independent of level of loading.

4.4.1 Effect of massage on immune system associated genes

We show in this study that 47% of the significant functional gene clusters obtained from gene ontology are associated with the immune response following four daily bouts of massage. The importance of this finding lies partly in the fact that we investigated uninjured muscle tissue, which indicates the muscle itself is capable of generating a large change in expression of immune related genes. It was previously shown that massage induced a beneficial immune response in patients with breast cancer [118,126,127], in children and adults with HIV [119,128,129], and in pre-term infants [122]. Moreover, genes involved in the immune system were the largest functional group changed in white blood cells as response to massage [130] and immune function was improved after either a single bout or repeated sessions in healthy individuals [121,123]. These human studies all indicate that the innate immune system is capable of responding to changes in mechanical activity exerted on muscles. Mechanotransduction is a powerful tool with regard to cell signaling: transferring mechanical energy into a chemical response results in quicker signal

transduction from the cell surface to the nucleus than that of a ligand-receptor interaction [90]. Manual therapies like massage utilize this principal, and according to our data, have a measureable effect on the expression of immune system-related genes. Recently it was shown that massage therapy attenuated pro-inflammatory signaling after exercise-induced damage [98], but to our knowledge the current study is the first to show changes in expression of immune-related genes in healthy uninjured muscle tissue.

4.4.2 Mechanodependency of inflammatory responses

The gene expression pattern analysis suggests a load dependent response to massage. Most genes remained relatively constant with low load (1.4N) but exhibited a change with moderate load (4.5N). We validated the gene expression pattern of four genes involved in the regulation of the immune system and the inflammatory response: CCR2 interacts with various pro-inflammatory cytokines to regulate cell chemotaxis as a surface cell receptor expressed in immune cells such as neutrophils and macrophages [131]. CCL2, also known as monocyte chemoattractant protein-1, is the primary ligand for the CCR2 receptor [65,66,132], and the CCR2/CCL2 interaction has shown to be a critical regulator of the autonomous inflammatory response in skeletal muscle [65,132]. Mice deficient in CCL2, as well as CCR2 receptor null mice, display a delayed inflammatory response that results in deficient regeneration [65,66,132]. CCR2 null mice also display a retarded inflammatory process characterized by diminished macrophage recruitment and adipocyte infiltration [65,132]. Thus these proteins are intricately involved in the inflammatory response of skeletal muscle. CD74 is a transmembrane protein expressed on antigen-presenting cells and is a receptor for macrophage inhibitory factor (MIF) [133,134]; LILRB4 is a leukocyte Ig-like receptor that is expressed on antigen presenting cells, displays an immune inhibitory nature, and has been suggested to play a role in regulating both the innate and adaptive immune systems [135]. LYZ2 is a lysosomal enzyme involved in lysosomal activities such as: reducing local expression of pro-inflammatory cytokines (tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6); interferon-gamma (INF-γ), interleukin-8 (IL-8), and interleukin-17 (IL-17)), while increasing the expression of anti-inflammatory cytokines (interleukin-4 (IL-4) and transforming growth factor-beta (TGF-β) [136]). The load-dependent alterations in the abundance of these genes may be a contributing factor to the

reduction of pro-inflammatory cytokines such as TNF-α and IL-6 observed in previous investigations [98], and ultimately influence immune cell accumulation.

Our histological data indeed suggest that cellular abundance is affected by the magnitude of compressive load applied, and is elevated at higher loads. Notably, in the HL condition there is a significant increase in M1 and M2 macrophages. M1 macrophages are considered to be phagocytic in nature [3-5,117] and are responsible for the removal of cellular debris resulting from the initial injury. M2 macrophages are resident in the tissue, classified as non-phagocytic, and serve to aid in repair and regeneration [1,3,8,117]. Macrophage phenotype is thought to be plastic and subject to influences from the microenvironment, such that they can undergo phenotype transition depending on the presence and/or absence of certain cytokines: pro-inflammatory cytokines such as IL-6, IL-8, and TNF-α promote the action of M1 macrophages, whereas anti-inflammatory cytokines (e.g. IL-4, TGF-β, and IL-10) induce the activation of M2 macrophages [1,9,10,117]. However, large quantities of M1 macrophages have been correlated with the severity of the immune response, and therefore the increase in the muscles subjected to high loads (11N) most likely signifies an actual injury to the muscle. Various clinical treatments utilize the application of high force (e.g. deep tissue, or cross-friction massage) to induce local inflammation, with the goal of promoting repair and regeneration. It is unknown at this time whether or not the pro-inflammatory response elicited by the application of high load massage in this study would be of benefit in those situations. Acute versus chronic injury environments differ considerably, and in some chronic situations inducing a local inflammatory response, elicited by high load application, may be of benefit.

Of note is that we did not observe an increase in neutrophil abundance with massage at any level of load. Neutrophil infiltration usually peaks between 6-12 hours following a perturbation and subsides within 24 hours [1,6,8]. Therefore, it was not surprising that we did not detect an abundance of neutrophils in the muscle tissue four days from the initial bout of massage. However, it has been suggested previously that neutrophil infiltration may not be a required component of the inflammatory process in certain types of skeletal

muscle injury (e.g. exercised induce damage) [124]. Hence neutrophils may not be modulated with massage application. The data from our study imply that the moderate load induces an immunomodulatory response largely mediated by macrophages, which is likely beneficial; however, high loads induce an inflammatory response indicative of damage.

4.4.3 Systemic effect of massage

An unexpected result from our study was the fact that massage induced a load-independent cellular immune response, in the contralateral leg. This mimics the cross-education (cross-over) effect of exercise in which the contralateral leg improves in performance even though only the ipsilateral leg is exercised [137]. With exercise this effect has been attributed to neurally mediated mechanisms residing at the level of the spinal cord [137]. It is possible that the cross-over effect of massage as observed here is also mediated through neuronal mechanisms; however, we suggest that it is mainly induced by an endocrine-like mechanism, because previous studies have shown that massage can induce changes in the number of circulating immune cells and their function [121,123]. We suggest that CCL2 produced by resident skeletal muscle macrophages is potentially responsible for the mobilization of monocytes from the bone marrow [65,132], and ultimately the recruitment of pro-inflammatory macrophages to the muscle tissue; massage application may enhance this effect.

4.4.4 Mechanical Considerations Regarding Massage Application

Previous studies have shown a load-dependent effect of massage on recovery of function after muscle damaging event [97], but to our knowledge this report is the first to show load-dependent changes in immune markers in uninjured muscle tissue. The advantage of our device is that loads can be carefully controlled, unlike in the human condition where loads are quite often not described, vary during the massage session, or are described in subjective terms such as ‘within the range of comfort’, ‘light or moderate pressure’ or ‘as tolerated’ [130,138-141]. With over 75 different methods associated with massage therapy [23] application of the treatment should be carefully considered based on the desired outcome. The present study most closely mimics a bidirectional, effleurage technique and it is

extremely difficult to infer whether or not a different mode of application, or combination of stokes, would have the same results.

4.5 Conclusion

When a massage mimetic is applied to healthy skeletal muscle, the inflammatory response is load dependent and associated with mechanosensitive regulation of various immune related genes. Here, we have also demonstrated a systemic response to compressive loading, resulting in an increase of M1 macrophages in the contralateral limbs. Although this study provides some preliminary insight into the physiological mechanisms behind massage in skeletal muscle, further studies are needed to establish the efficacy behind its use. Immunomodulatory properties of massage make this manual therapy an attractive alternative intervention to pharmaceuticals for various inflammatory conditions. As massage is increasingly integrated into conventional medicine, determining the appropriate parameters for application will become increasingly important to maximize beneficial outcomes.

4.6 Acknowledgements

The authors would like to acknowledge Sarah Abshire, Amy Confides, Jyothi Mula, Dr. R. Grace Walton, and Dr. Tommy Cunningham for technical assistance, in addition to Dr. Arnold J. Stromberg and Yanling Hu for statistical instruction. Research reported in this publication was supported in part by the National Heart, Lung and Blood Institute (NHLBI) of the National Institutes of Health under the institutional training grant T32 HL086341, Research Training in Muscle Biology of the Cardiopulmonary System, National Athletic Trainers Association (NATA) Osternig Grant, National Athletic Trainers Association (NATA) Foundation Research Grant, and the University of Kentucky College of Health Sciences pilot grant. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

CHAPTER FIVE: CROSSOVER EFFECT OF MASSAGE FOLLOWING ECCENTRIC EXERCISE INFLUENCES ABUNDANCE OF MACROPHAGE POPULATIONS AT SIX HOURS

5.1 Introduction

The inflammatory response is a highly conserved and critical component required for protection against invading pathogens, rogue, maladapted cells, and in the case of skeletal muscle, essential to the repair and regeneration of tissue following damage or injury[28,142]. A diminished response of the cells comprised of the innate immune system (e.g. neutrophils, macrophages) has shown to dramatically hinder the repair of skeletal muscle marked by an increase in necrotic tissue and intermuscular lipid[12,65]. Conversely, the inflammatory response and its cellular components have been shown to further exacerbate initial tissue damage in acute conditions of muscle injury[55]. Evidence has shown in the acute stages of injury, peaks in neutrophil infiltration, are responsible for production of cytotoxic chemicals, at six hours post injury[1,3,6,55]. Macrophages, responsible for phagocytosis (M1) and repair (M2), enter the tissue at 24 and 48 hours respectively[1,3,6,55].

Pharmaceuticals, such as non-steroidal anti-inflammatory drugs (NSAIDs), are often used for the modulation of the inflammatory response. However, in skeletal muscle, NSAIDs that inhibit prostaglandin synthesis through cyclooxygenase (COX) pathways, have been shown to have detrimental effects[12]. Administered in animal studies, these drugs severely delay the repair process with reductions of cross-sectional area 22-33% three weeks following freeze injury[12], and significantly decrease MyoD expression; a myogenic regulatory factor that is crucial to skeletal muscle regeneration[143]. Establishing alternative treatments to aid the modulation of the immune response without impeding regeneration would be favorable.

Massage is an alternative complementary medicine often sought after for the relief of various musculoskeletal maladies[25,34]. Although scientific evidence for its efficacy has been limited, a recent series of animal studies has demonstrated large changes in muscle

function by applying a massage mimetic once a day for four-consecutive days immediately after damaging eccentric exercise. The investigators noted enhanced functional recovery of force[19,97]; recovery of passive mechanical properties such as stress relaxation and creep[26]; and a noticeably diminished infiltration of leukocytes/monocytes [19] in the massaged versus the non-massaged, exercised contralateral limb. Interestingly, these beneficial effects of massage were reduced if the massage-mimetic was not applied immediately following exercise, and were completely abrogated if delayed 24 hours[20]. Because the innate cells of the immune system respond to injured tissue in a well-orchestrated, temporal fashion following injury,[1] modulation of this response is a potential result of massage application. Recently, pro-inflammatory cytokine levels of tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) were decreased in muscle biopsies taken from human subjects receiving massage application to exercised quadriceps muscles[98] However, no direct quantification of the inflammatory response was performed.

These recent studies have significantly contributed to our understanding of the effects of massage application on exercise induced damaged skeletal muscle. However, methodological limitations fail to elucidate the effects of a single bout of massage on the response of innate immune cells over the time course of acute inflammation following eccentric exercise. Therefore the aims of this study were to i) determine if a systemic effect of massage exists over the early phases of the inflammatory response to damaging eccentric exercise, and ii) if a single bout of massage has the ability to produce a beneficial shift in the inflammatory response, potentially resulting in the reduction of inflammatory cell infiltration and early recovery of force production in skeletal muscle following eccentric exercise in rats.

5.2 Methods

5.2.1 Animals

Forty-eight Long Evans rats (400g, Harlan Laboratories, Indianapolis, IN) were used in this study. Rats were housed in cages within the animal housing facility at the University

of Kentucky with access to food and water ad libitum. All procedures were approved by the University of Kentucky's Institutional Animal Care and Use Committee.

5.2.2 Experimental Groups

Changes in torque production and cellular infiltration following eccentric exercise and massage, were evaluated for the tibialis anterior (TA) muscle *in-vivo*. To evaluate the temporal nature of the acute inflammatory response we chose three time points to capture previously established[1] peaks of inflammatory cells: 6-hours reported as the typical peak of neutrophil infiltration; 24-hours reported as the typical peak of M1 phagocytic macrophage infiltration; and 12-hours to evaluate any potential shifts in this time course. Various control conditions were required to assess: basal levels of resident macrophages; the effects of surgical intervention; and a control to record the natural time course of the inflammatory response following exercise in addition to a potential systemic effect of massage. Rats were randomly assigned to one of eight groups: Control group (n=6) which received no surgical intervention, no massage, and no exercise; Sham Control group (n=6) which served as a surgical control to evaluate potential surgical induction of an inflammatory response. Three Eccentric Exercise Control groups at each time point (6hr EEXCon n=6, 12hr EEXCon n=5, 24hr EEXCon n=6); and Eccentric Exercise + Massage groups at each time point (6hrs EEX+MASS n=5, 12hr EEX+MASS n=5, 24hr EEX+MASS n=5) of which both limbs underwent eccentric exercise but received massage to the *right* limb only, leaving the left limb to serve as an internal exercise control. A 9th group, 1-hour EEXCon time point (n=3) was collected to assess early infiltration of neutrophils histologically. Two animals from the 1hrEEXCon group were subjected to the same exercise protocol, while one rat performed treadmill running (15m/min) downhill (-16%grade) with intermittent rest periods. To encourage running animal was electrically stimulated by an electrical shock grid placed at the end of the belt (Columbus Instruments, Columbus Ohio). Due to inherent surgical complications, animals that experienced nerve damage resulting in atrophy, or lack of maximal force production were not evaluated mechanically or histologically. Animals that exhibited co-contraction of the agonist triceps surae muscle group were eliminated from the mechanical data but were evaluated histologically as the tibialis anterior muscle was

likely subjected to sufficient eccentric exercise. One animal died under anesthesia. In total 30 animals were used to analyze mechanical data, and 44 animals were used to analyze histological data.

5.2.3 Surgical Intervention

Animals comprised of the Sham group, EEX Control groups, and EEX+MASS groups were anesthetized via isofluorane. A small incision was made over the lateral portion of the biceps femoris muscle at the tibial plateau. The biceps femoris muscle was then separated to expose the underlying peroneal nerve. Custom made nerve cuff electrodes were secured around the peroneal nerves of both limbs. With the exception of the Sham control, the wires of the nerve cuff were routed under the skin using blunt dissection to the base of the skull, which was exposed and cleaned. Wires were they were soldered to a Amphenol 12-pin connector (Samtec), secured with dental cement (Perfex Denture Repair Powder, International Dental Products) and four stainless-steel screws (Fine Science Instruments) **(Figure 5.1).** The incisions were sutured, and all rats received buprenorphine (0.02 mg/kg, subcutaneous injection) immediately following surgery and at every 12 hours for 48 hours. Animals were returned to their individual cages to recover for one week. Sham control animals were euthanized after one week to assess potential effects of the surgical intervention on the inflammatory response in the tibialis anterior muscle.

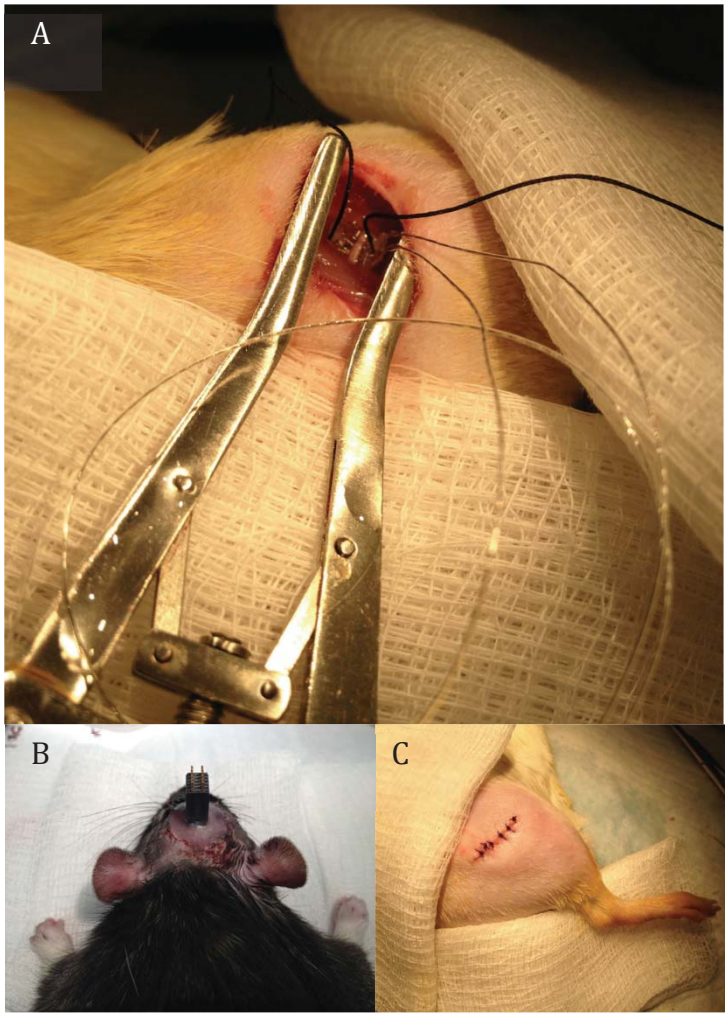


Figure 5.1: Surgical Instrumentation

(A) Surgical implantation of custom nerve cuff electrode on the peroneal nerve, distal to bifurcation of the sciatic nerve (both limbs were instrumented). **(B)** Wires from nerve cuff electrode were routed under the skin to the base of the skull where they were soldered to a head connector secured to the skull with four screws and dental cement. **(B)** Closed incision site.

5.2.4 Exercise Protocol

One week following surgical implantation of nerve cuffs, animals were anthestized with isofluorane and were placed laying prone in a sling hammock with both legs passed through limb holes. The left foot was then secured to a footplate connected to a torque sensor on the cam of a servomotor **(Figure 5.2)**. Using a goniometer the tibiotarsal angle was set to 90°. The head-connector was plugged into the output channel of an electrical stimulator (Grass S88X, Grass-Telefactor), the α-motor neuron threshold was determined and recorded. Before the commencement of exercise an isometric torque-joint angle relationship was established (to determine peak torque production) by supramaximally stimulating at 2-3x the α-motor neuron threshold at a tibiotarsal angle of 85° and progressing in increments of 5° to 130° for a total of 10 measurements.

The foot was returned to the resting position of 90° for 2-minutes between each measurement to eliminate factors of fatigue. Following the torque-joint angle relationship, the TA muscle was subjected to one bout of eccentric exercise, which consisted of seven sets of ten repetitions (with two minutes rest between each set). The tibialis anterior is stimulated to contract and held isometrically for 100ms, prior to a 500ms eccentric contraction of the dorsiflexors as the foot plate is moved 50° at 100 deg/sec from a tibiotarsal joint angle of 85° to 135°. Immediately following eccentric exercise the same limb underwent a second torque-angle relationship to record the reduction in torque post exercise from the baseline. The left leg was removed from the platform and the process was repeated for the right limb. Prior to sacrifice, each animal was subjected to a third and final torque-joint angle relationship to assess the recovery from eccentric exercise, and the final torque reduction at time of euthanization.

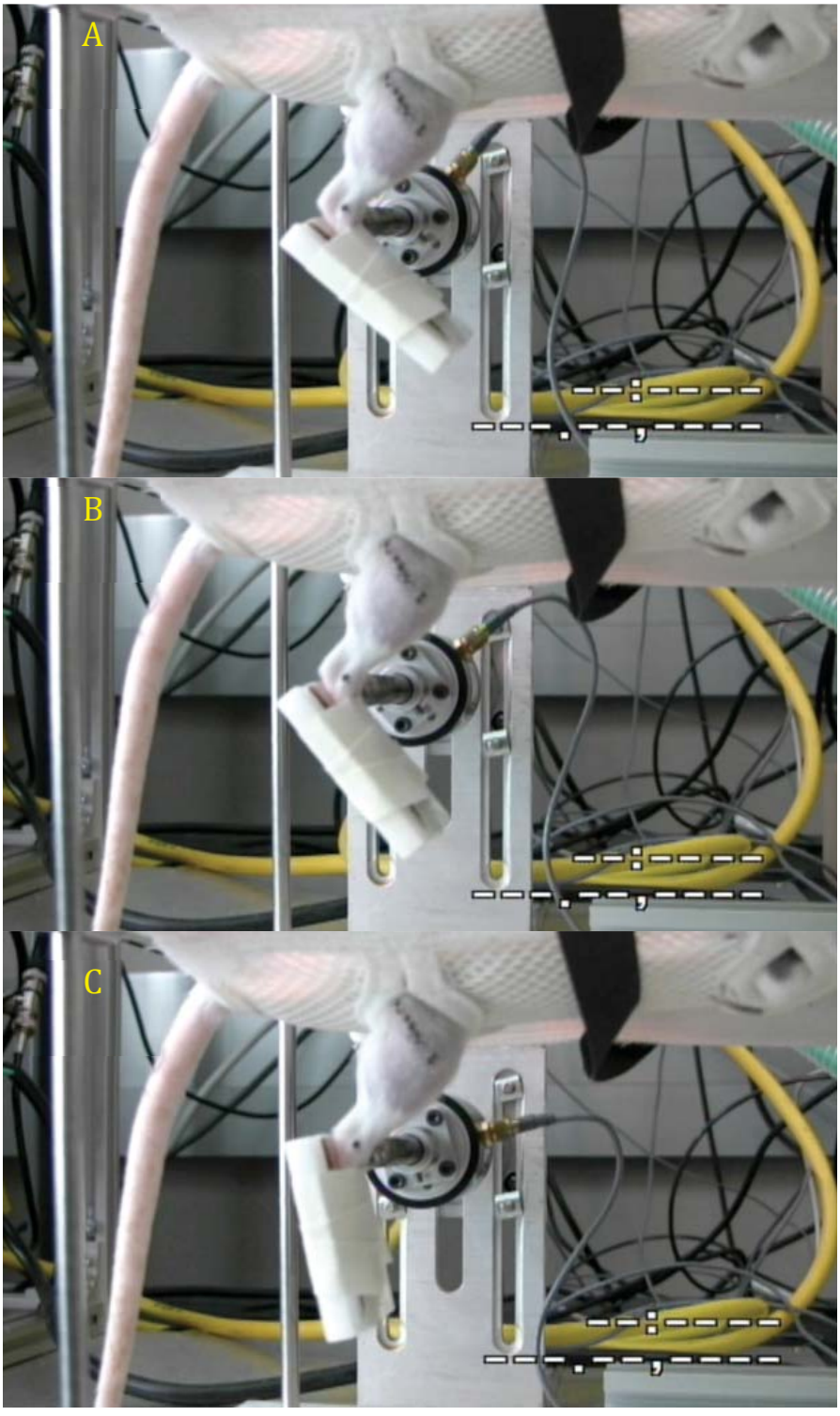

A
B
C

Figure 5.2 (Continued): Eccentric Exercise

(A) Starting position for eccentric exercise: Ankle at 90° relative to knee joint. **(B)** Tibialis Anterior is stimulated to contract and held isometrically for 100ms, prior to a 500ms eccentric contraction of the dorsiflexors as the foot plate is moved to 140° at 100 deg/sec. **(C)** Terminal plantar flexion and deactivation as the footplate is returned to the starting position of 90°.

5.2.5 Massage Protocol

Immediately following the collection of the post-exercise torque-joint angle relationship, the anesthetized rats were immediately turned lateral recumbent with the anterolateral portion of the right TA muscle facing superiorly. Utilizing our previously established custom cyclic compressive loading device **(Figure 5.3)**[19], the right tibiotarsal joint was secured to a platform and the tibialis anterior muscle was subjected to a single 30-minute bout of cyclic compressive loading along the length of the muscle (cycling 15mm distal to proximal, proximal to distal at an application load of 4.5N, and a frequency of 0.5Hz) Loading conditions were determined through pilot work and previous reported optimal load determined for a rabbit model, scaled to a 400g rat[19]. EEXCon rats did not receive massage. Following massage application the rats were returned to their cages until euthanized at each of their respective group time points.

5.2.6 Histology

5.2.6.1 Hemotoxylin and Eosin

Animals were euthanized at their respective time points following the collection of the final torque-joint angle relationship. The TA muscle was harvested, flash frozen in liquid nitrogen, and cryogenically preserved for evaluation. A section taken from the belly of the muscle was mounted on to a cutting platform using OTC medium, and muscle cross-sections were cut at 8μm; every fifth section was fixed using methanol (100%) and reacted for standard hematoxylin and eosin staining. A total of four sections per muscle were analyzed.

5.2.6.2 Immunohistochemistry

TA muscle sections were cut as stated above and fixed in ice-cold acetone (100%). Sections were blocked in 3% H_2O_2 in PBS, followed by normal horse serum (ImmPRESS-Vector Laboratories Burlingame, CA). Each tissue section was reacted for three primary antibodies that were each incubated overnight: i) Neutrophils: Mouse anti-Rat CD43; ii) M1 macrophages: $ED1^+$ Mouse anti-Rat CD68; and iii) M2 macrophages: $ED2^+$ Mouse anti-Rat CD163 (Serotec Raleigh, NC).

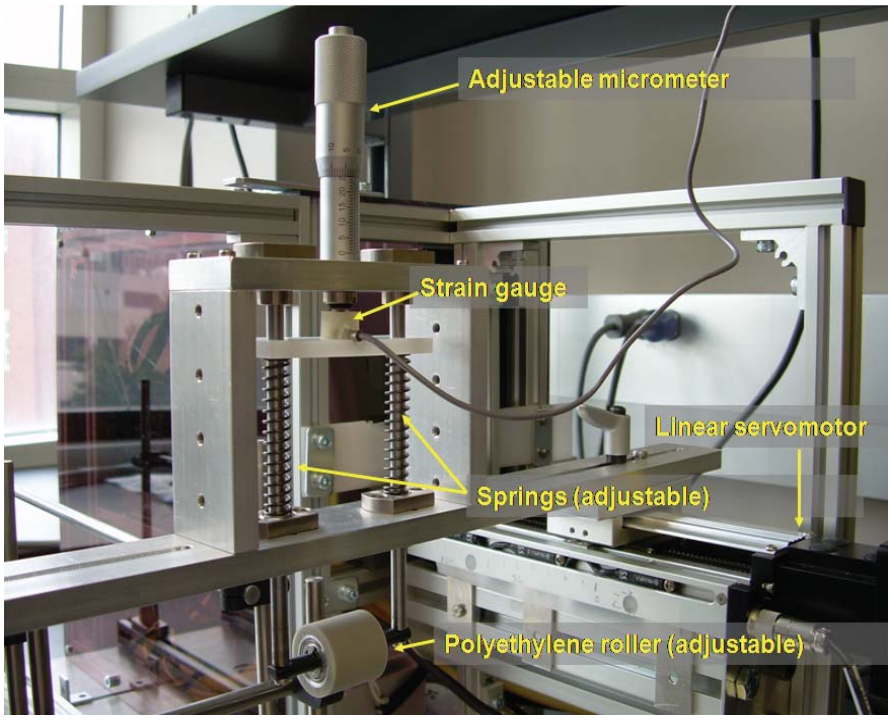


Figure 5.3: Massage Mimetic

Custom fabricated **CCL device** has the ability to quantify compressive loads to soft tissues during cyclic motions, from a range of 1.4N to 100N[5] utilizing a spring loaded roller mechanism. Vertically mounted micrometer allows precise adjustment of the compressive load applied to two springs arranged in parallel with a known Hooke's spring constant, to allow accurate calibration of forces applied through the 25mm roller with urethane coating of a known hardness. Linear translation, stroke length, and frequency were controlled by a servomotor and linear actuator providing positional feedback and data in real time

ImmPRESS anti-Mouse IgG (*Vector Laboratories*) secondary antibody with fluorescein (for M1), cyanine-3 (Cy3 for M2) and cyanine -5 (neutrophils) was applied in amplification buffer (Tyramide Signal Amplification (TSA) *Perkin Elmer Waltham, Mass.).* Tissue was blocked withVector® Mouse-on-Mouse (M.O.M) Ig blocking reagent and normal horse serum (NHS) in between primary antibody applications. 4'6-diamidino-2-phenoylindole (DAPI) was applied at 0.01μM to visualize nuclei. All histology images were obtained using a Zeiss Axio Imager M1 microscope (*Carl Zeiss Microimaging GmbH Göttingen, Germany)*

5.2.6.3 Sterological Point Counting

Total cellular abundance was measured using a random stereological point counting technique. Using a Zeiss Axio Imager M1 microscope at 100x magnification, one randomly selected field from each of the four sections per muscle was photographed and used for cell counting. Sections were selected blindly by turning the monitor off, moving the microscope stage to a section, turning on the monitor, and then capturing the image. Edges of the muscle section were not captured, nor were any areas with noticeable cutting/freeze artifact. Cells in the interstitial space outside muscle fibers were counted as total cellular abundance on the H&E slides. For determination of M1, M2, and neutrophil cellular abundance, we counted cells that were positive for primary antibody and also reacted with DAPI. Due to the pleiotropic nature of macrophages, cells that were reacted for both M1 and M2 antibodies, in addition to DAPI were counted as transitioning macrophage cells. Cell counts from each of the four sections per muscle were averaged, and expressed as cells per field of 0.60mm^2.

5.2.7 Statistical Analysis

5.2.7.1 Mechanical Data

Mean torque at each joint angle was assessed for each of the torque-joint angle relationships for each individual animal, and limb respectively. Peak torque at baseline prior to exercise, peak torque immediately following exercise bout, and final peak torque before sacrifice were recorded to provide three values of interest: reduction of torque post

exercise, recovery of force from post exercise values, and final torque reduction. Independent sample t-tests were used to compare the EEXCon vs. the non-massaged left limb of the EEX+MASS animals to assess a crossover effect of massage on torque production over time. Paired sample t-test were used to compare the non-massaged left limb and the massaged right limb of the EEX+MASS animals to assess the effect of massage on torque production over time.

5.2.7.2 Histological Data

Mean abundance of cells, number of muscle fibers per 0.60mm^2 area for both H&E and immunohistochemistry were recorded, in addition to whole muscle wet weights, for each animal.

All statistical analyses were performed using IBM SPSS 19.0 (SPSS Chicago, IL). Graphs were generated using SigmaPlot (Systat Software Inc. San Jose, CA). For all parameters measured, mean and standard error are reported. Due to the inherent biological variability of cellular response over time, immune cell data for specific cells M1 and M2 macrophages were transformed using the natural log (Ln) function. Levene's statistic for was used to detect violations in homogeneity of variances. Independent t-tests were utilized to compare differences between control groups to determine existence of a crossover effect. Student's paired t-tests were used to determine difference in cell numbers between the massaged limb and non-massaged contralateral limb. One-tailed p-values are reported. One-way ANOVAs with were utilized to detect difference between time points for each limb respectively. Tukey HSD post-hoc analyses were used to compare significant differences. Due to inherent biological variability significance was set at *a priori*= 0.10.

5.3 Results

5.3.1 Lack of Inflammatory Response with Surgical Intervention

Each animal was given one-week to recover from surgical intervention before undergoing an exercise protocol. Sham controls were used to determine whether or not the surgical intervention induced a significant inflammatory response in the TA muscle.

Comparisons of the Sham versus the Control group found no significant differences in general cellular infiltration (determined by H&E), M1, M2, neutrophils, or transitional macrophages **(Figure 5.4)**.

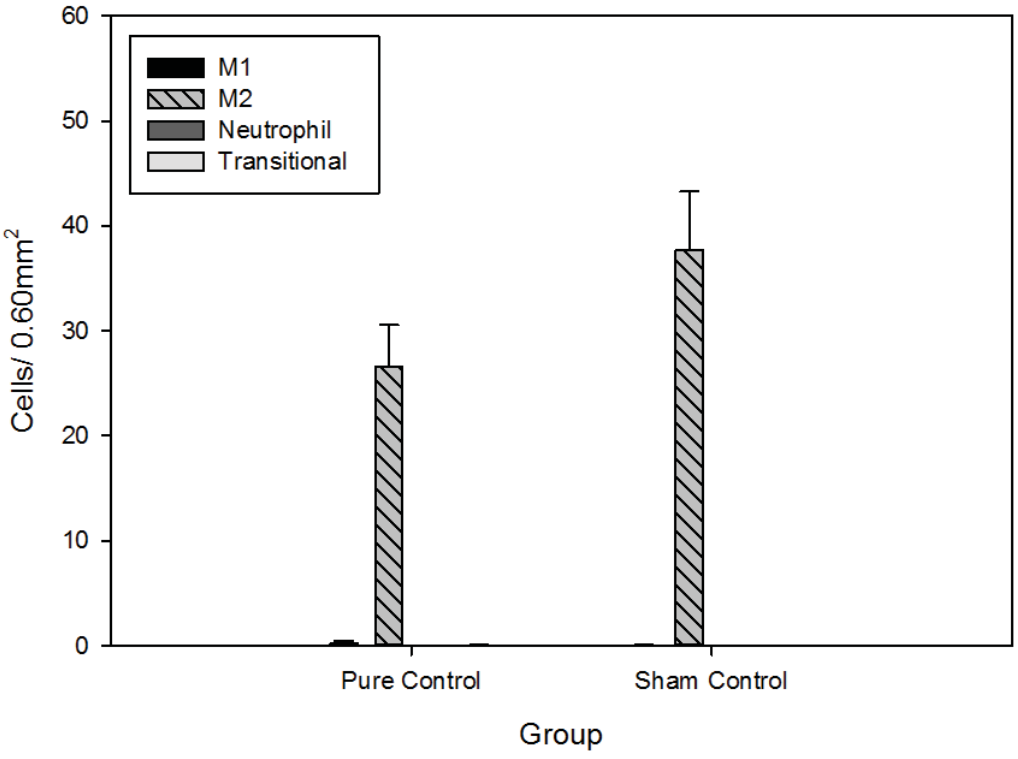


Figure 5.4: Surgical treatment did not induce inflammatory response in TA muscle

Comparison of the pure control condition versus the sham control. No significant differences in neutrophils, transitional macrophages, M1, or M2 macrophages were observed between the two groups.

Together, these data indicate our surgical intervention, accompanied by a one-week recovery period, do not promote a change in the inflammatory environment of the TA muscle. Additionally, no differences in muscle wet weights or fiber number per cross-sectional area were detected across all groups analyzed **(Figure 5.5 A and B)**.

5.3.2 Crossover Effect of Massage Detected at 6hrs Following Exercise

Significant increases in general cellular infiltration, and M1 abundance were detected at each time point across all exercise groups compared to the Control condition. Comparisons of the EEXCon groups versus the non-massaged left limb of the EEX+MASS group revealed a crossover effect of massage in left limb at the 6hr time point. General cellular infiltration **(Figure 5.6A)**, and M1 macrophages **(Figure 5.6B)** were found to be greater in the EEXCon group versus the non-massaged left limb in the EEX+MASS group. Conversely, M2 macrophages were detected at significantly higher levels in the same non-massage limb of the EEX+MASS group compared to the EEXCon group (**Figure 5.6B**). Comparison of the EEXCon limbs against the right limb of the EEX+MASS group at 6hrs, showed no significant difference between the EEXCon and the massaged limb for general cellular infiltration M1, or M2 macrophage abundance (**Figure 5.6 A&B**). However, when the massaged and non-massaged limbs are compared against each other, the massaged limb has an increased abundance of infiltrating cells (H&E) and M1's, coupled with a decrease in M2 abundance versus the non-massaged limb **(Figure 5.7)**. Together, these data suggest a crossover effect of a single bout of massage applied following eccentric exercise that presents as early as 6hrs post exercise.

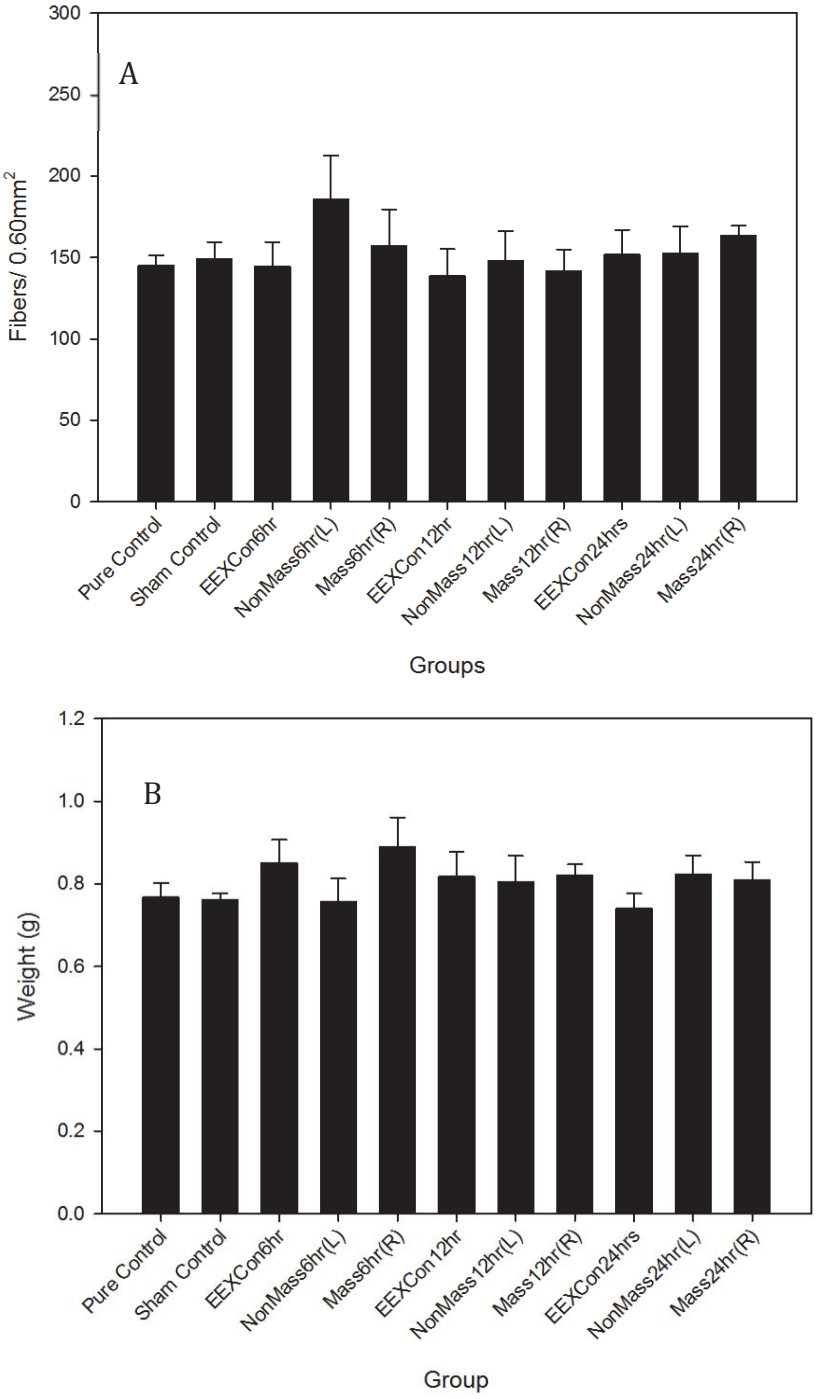
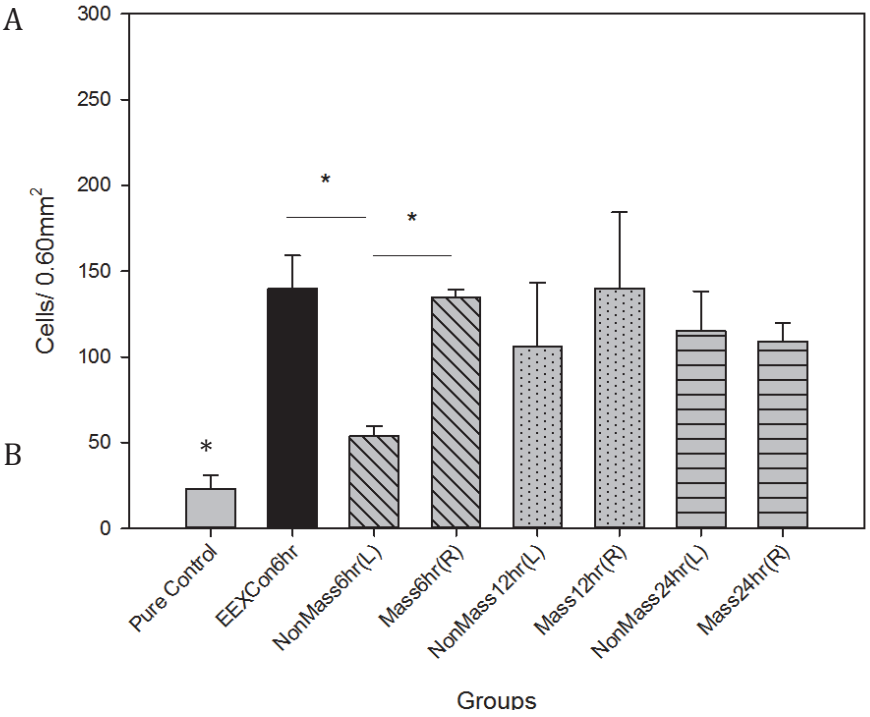


Figure 5.5: No difference in fiber number and weight.

No significant differences were detected across all groups for average number of fibers per cross-sectional area **(A)** or wet weight recorded at harvest **(B)**.

A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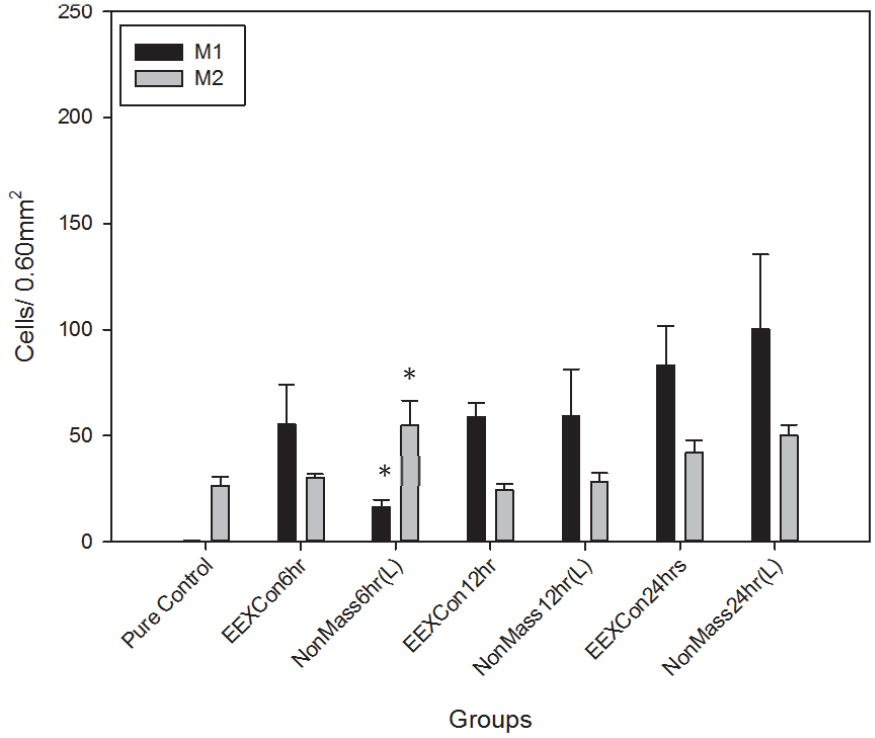
Groups

B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Pure Control
EEXCon6hr
NonMass6hr(L)
EEXCon12hr
NonMass12hr(L)
EEXCon24hrs
NonMass24hr(L)
Groups

Figure 5.6 (Continued): Crossover effect present at 6hrs post exercise

Comparison of general cellular infiltration across all groups resulted in a significant difference in cellular infiltration versus the pure control group (* over pure control group used for convenience), and the non-massaged limb versus both the exercise control and massaged conditions (A). This potential crossover effect at 6hrs was corroborated with the comparison of the exercise control groups versus the non-massaged limbs for M1 and M2 macrophages (B).

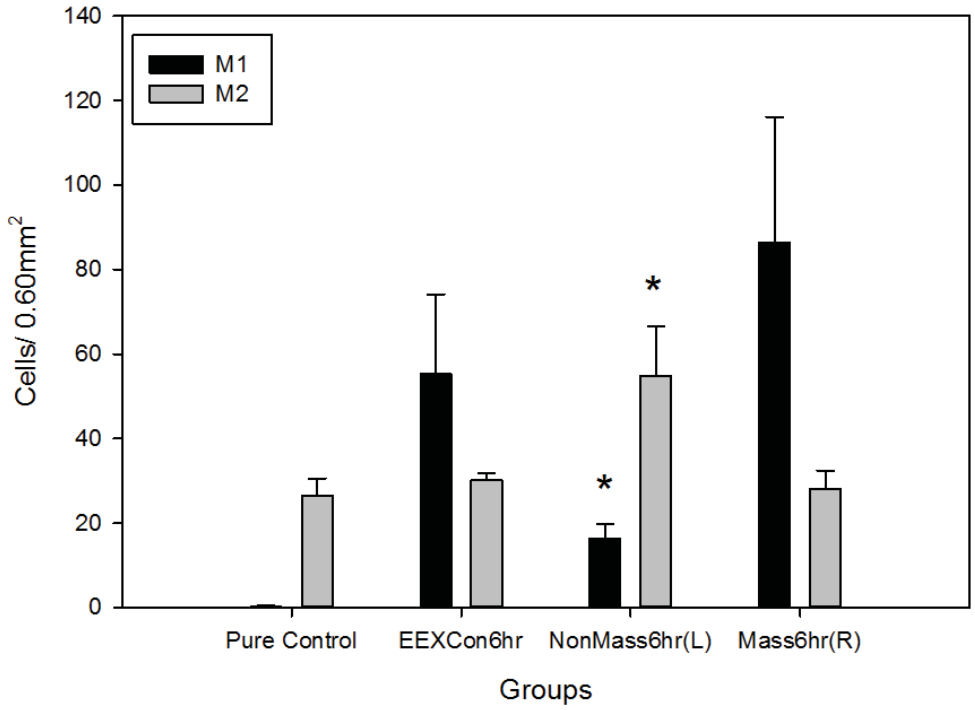


Figure 5.7: Massage promotes increase in M2 macrophages and a decrease in M1 macrophages in the opposing limb at 6hrs.

Evaluation of the crossover effect at 6hrs. The non-massaged limb in the 6hr group displayed a markedly lower abundance of M1 macrophages, and a higher abundance of M2 macrophages versus the exercise control and the massaged limb. No significant differences exist between the massaged limb and the exercise control limb.

5.3.3 Immune Cell Abundance

Although differences were not detected between massaged and non-massaged limbs at the 12- and 24hr time points, comparisons between each respective limb revealed significant differences in abundance of M1 and M2 macrophages across time points (**Figure 5.8)**. In the non-massaged limb M1 abundance was greatest at 24hrs, in addition to being higher at both the 6hr and 12hr time points compared to the control condition. M2 macrophages were elevated at 6hrs and 24hrs versus the control group, but reduced at 12hrs versus the 6hr group in the non-massaged limb. In the massaged limb, M1's were elevated at all time points compared to the control condition, and M2 macrophages were only significantly elevated at the 24hr time point versus the control condition. Differing time points at which macrophages peak in abundance relative to massage application, may signify a shift in the inflammatory response **(Figure 5.9)**.

5.3.4 Lack of Neutrophil Infiltration in Early Stages of Inflammation

Neutrophil staining was rare in the TA muscle sections for all conditions. Concerned that neutrophils may migrate to the site of injury sooner than 6hrs, we collected three animals 1hr post exercise. The average torque reduction post exercise for the two rats that underwent the standard eccentric exercise protocol was 0.65±0.10%. Torque could not be evaluated in the downhill treadmill rat. Histological evaluation of this tissue was unremarkable, and no neturophils were detected in the tissue at one-hour following eccentric exercise. Suggesting that neutrophils may not be required for sterile muscle repair and regeneration following eccentric exercise.

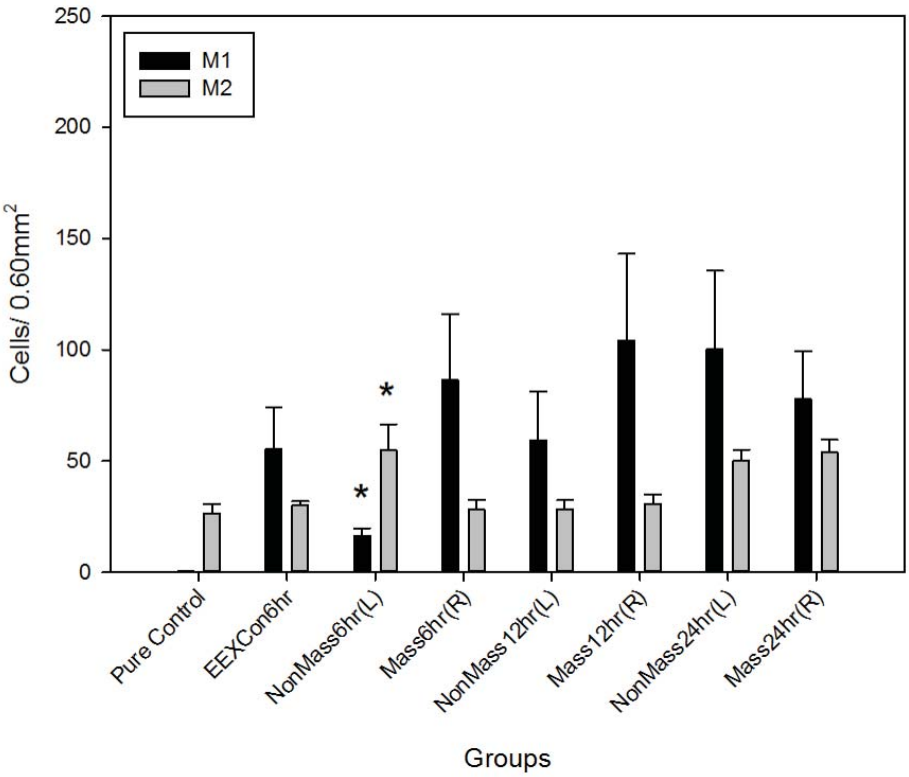
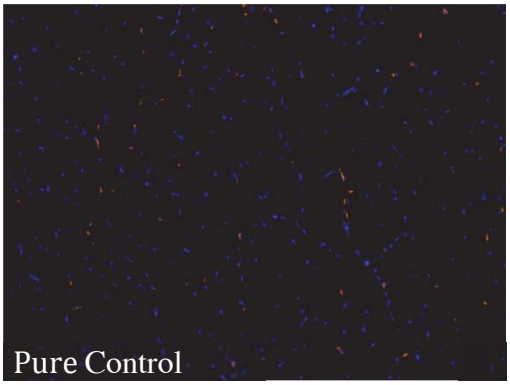
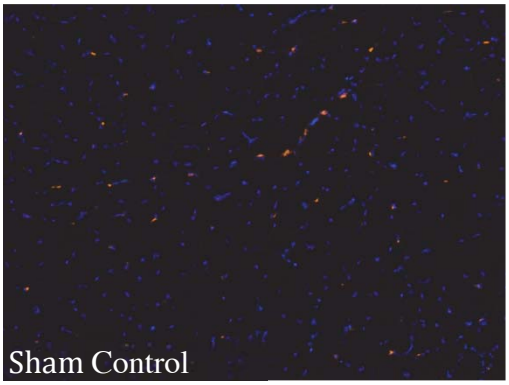
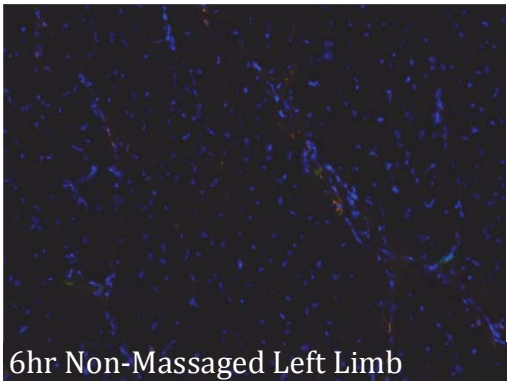


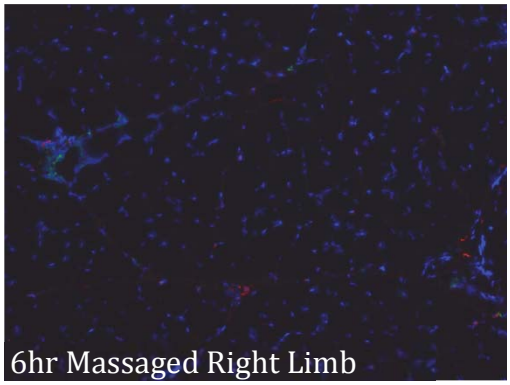
Figure 5.8: Modulatory effects of massage appear to take place early following massage application

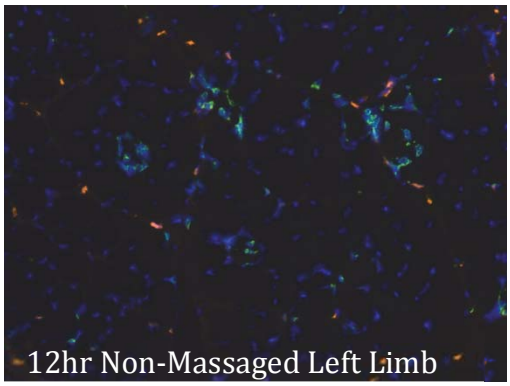
Comparison of M1 and M2 macrophages across all time points (right vs left limb), the crossover effect at six hours is displayed against the 6-hour exercise control group. Figure displays a peak in M1 macrophages at 12hrs in the massaged limb, and a peak in M2 macrophages at both 6hrs and 24hrs post exercise.

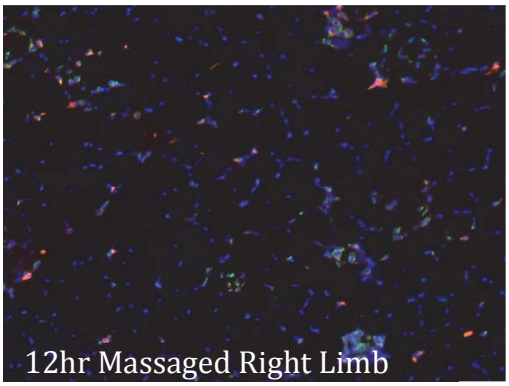

Pure Control

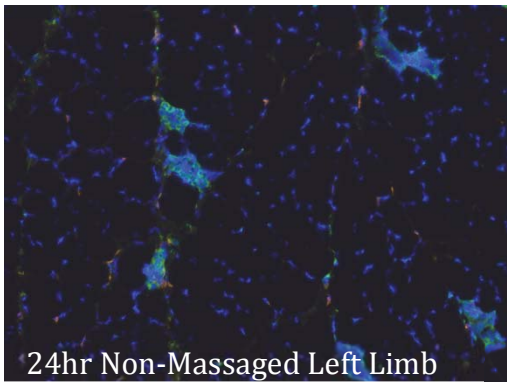

Sham Control


6hr Non-Massaged Left Limb


6hr Massaged Right Limb


12hr Non-Massaged Left Limb


12hr Massaged Right Limb


24hr Non-Massaged Left Limb

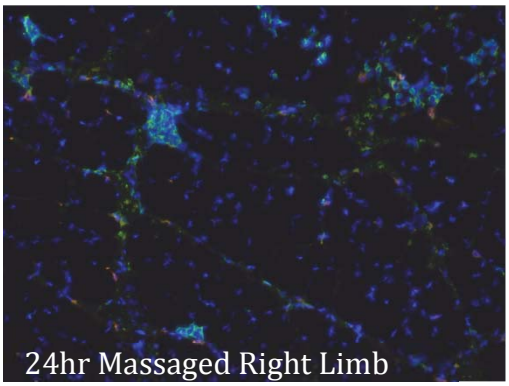

24hr Massaged Right Limb

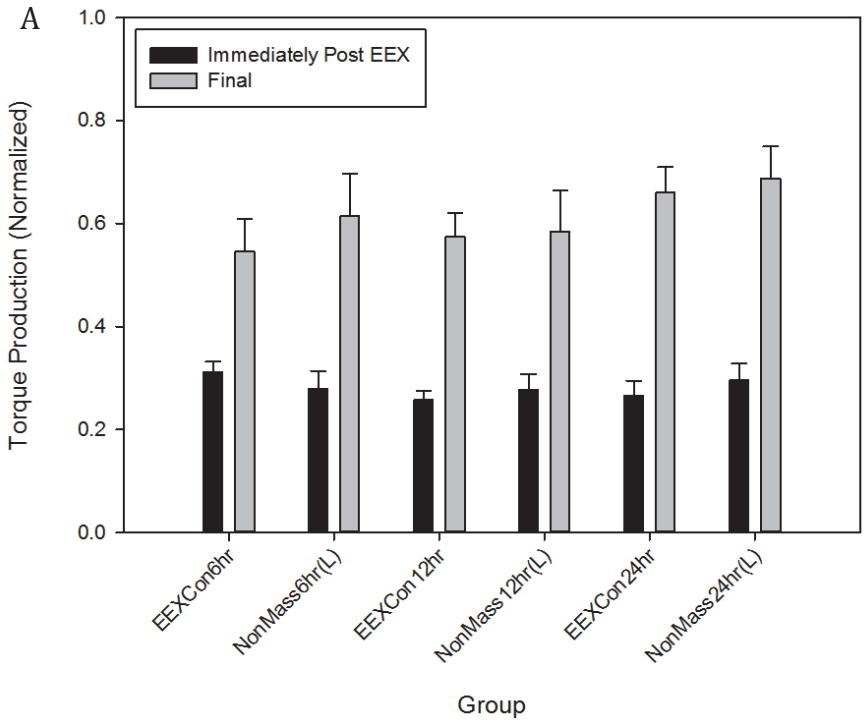
Figure 5.9 (Continued): Immunohistochemistry

Representative immunohistochemistry stains for each time point. Green are M1 macrophages (CD68), orange are M2 (CD163) macrophages, Magenta is neutrophil (CD43), and blue is DAPI. Note positive staining of M2 resident macrophages in the Pure Control and the Sham Control conditions. Images are taken at 100x.

5.3.5 Mechanical Data

Average reduction of torque immediately post eccentric exercise was 0.71± 0.01% for the animals analyzed across the EEX groups, indicating our exercise protocol successfully induced a significant inflammatory response, and a reproducible reduction in torque reduction (**Figure 5.10 A&B**). Comparisons of the EEXCon groups and the non-massaged left limb of the EEX+MASS groups yielded no differences in reduction of torque: immediately post eccentric exercise; recovery of torque production from post eccentric exercise values; and final torque production before euthanasia versus baseline values (**Figure 5.10A)**. These data suggest that there is no crossover effect with massage in relation to torque production at these time points. Further comparisons between the left and right, as well as the time points within each limb, did not result in any significant findings **(Figure 5.10B)**. However, there appears to be trend present in the final torque production data in regards to a recovery in force production at 6hrs, a subsequent drop at 12hrs, and an improvement at 24hrs. The diminished force production at 12hrs coincides with the increased abundance of M1 macrophages in the non-massaged limb. Additionally, final torque production appears greater at 24hrs, which coincides with a peak in M2 macrophages. Failure to elucidate significance with our mechanical data was likely due to a type II error. Increasing the number of animals, hence improving our power, may result in significant correlations.

A


Immediately Post EEX
Final
Torque Production (Normalized)
1.0
0.8
0.6
0.4
0.2
0.0
EEXCon6hr
NonMass6hr(L)
EEXCon12hr
NonMass12hr(L)
EEXCon24hr
NonMass24hr(L)
Group

B

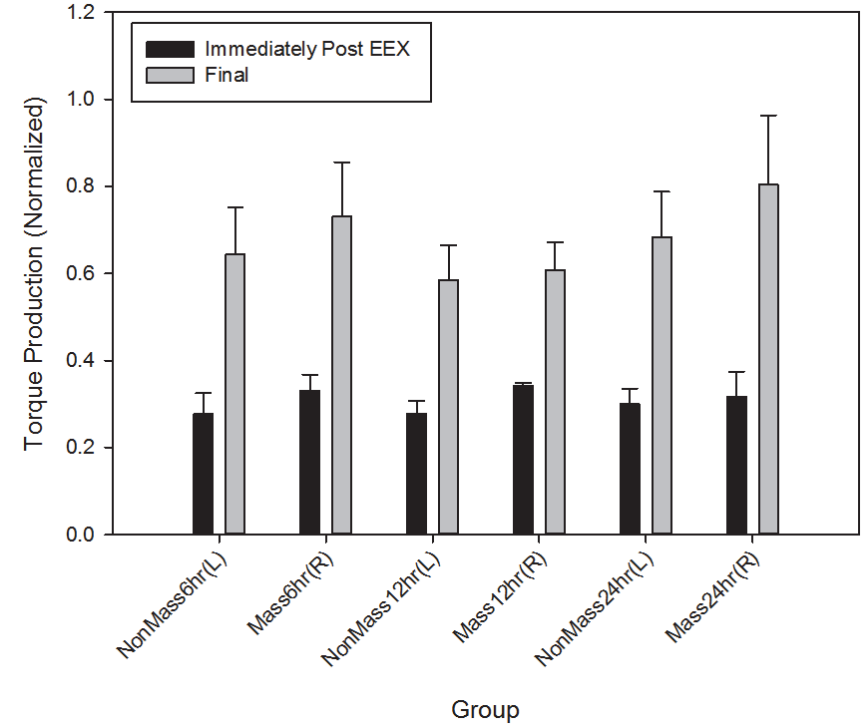

Immediately Post EEX
Final
Torque Production (Normalized)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
NonMass6hr(L)
Mass6hr(R)
NonMass12hr(L)
Mass12hr(R)
NonMass24hr(L)
Mass24hr(R)
Group

Figure 5.10: No differences in force production or crossover effect in final torque production as a result of massage (Continued).

Exercise control groups compared to the non-massaged limb resulted in no significant differences suggesting no crossover effect of torque production with massage **(A)**. **(B)** Right and left comparisons across time points yielded no significant differences.

5.4 Discussion

5.4.1 Immunomodulation

The first aim of our study was to determine if a single bout of massage has a systemic effect over the early phases of the inflammatory response. Our results suggest that massage does indeed have a crossover effect that was detected at 6hrs-post damaging eccentric exercise. A single 30-minute bout of massage to the right limb effectively diminished the abundance of M1 macrophages, and increased the abundance of M2 macrophages, in the non-massaged left limb when compared to the exercise control and the contralateral massaged right limb.

The markedly lower abundance of the M1 population in the non-massaged limb coupled by a significant increase in M2 macrophages is not the typical distribution reported in the early stages of inflammation[1,3,8,55,73]. This suggests that massage potentially manipulated the microenvironment in which these cells interact. Curiously, this was not a result of direct mechanical manipulation of the underlying tissues. Instead this appears to have taken place by some systemic mechanism. M1 macrophages can become chief producers of pro-inflammatory cytokines (such as TNF-α, and IL-1β)[80] in the early stages of inflammation. Attenuating the production of these cytokines through the reduction of their abundance in the early hours following injury, could beneficially modulate the inflammatory response from there after, promoting early transition into the repair and regeneration phase of healing. Considering that general cellular infiltration accessed through H&E staining also saw a significant decrease in cells at this same time point, suggests that less inflammatory monocytes were recruited to the skeletal muscle following exercise, and perhaps those that were, were influenced to transition to an M2 phenotype. This effect at such an early time point may explain why in previous work[20] massage is most effective when applied immediately after exercise as opposed to delaying the application for 48 hours. However, the crossover effect was not evaluated.

The pleiotropic plasticity of macrophages[10] makes them an attractive target for manipulation. Investigations have shown that macrophages do not likely terminally

differentiate but instead adapt and change to meet the need of their environment, including the ability to reverse[10]. The obscurity however, of neutrophils in this investigation was quite surprising. Data would suggest that any modulatory effect massage may have on the inflammatory response in skeletal muscle is likely through the manipulation of macrophage populations rather than the attenuation of the neutrophilic response. The reported presence of neutrophils following skeletal muscle damage in the literature is inconsistent[78]. Some of the most popular assays include: increased myeloperoxidase (MPO) activity, and antibody labeling[78]. Although neutrophils do produce MPO, other cells such as monocytes and macrophages produce MPO as well[78,145]. To complicate further, many of the antibodies used for the detection of neutrophils target antigens that are also displayed on monocytes, macrophages, and dendritic cells[79].

We stained our tissue with CD43 antibody that has been reported to have specificity for neutrophils in rat skeletal muscle[4,146]. Lacerated muscle tissue used in our lab as a positive control resulted in the staining of numerous neutrophils at 6hrs post laceration. Our data is consistent with LaPointe et al[124], who reported a lack in neutrophils following eccentric exercise in Wistar Rats, suggesting that the neutrophilic response may not be required for repair and regeneration following eccentric exercise.

A second consideration regarding neutrophils following muscle injury is the method in which injury is induced. Studies regarding the inflammatory response in skeletal muscle often use superphysiologic methods that impose unrealistic demands on the tissue (e.g. synergist ablation, hindlimb suspension/reloading); or ablate resident structure that may be critical in regulating the immune response (e.g. cardiotoxin injection); and potentially induce numerous pathogens to the area (e.g. freeze injury, cardiotoxin, endotoxin). Exercise protocols that require the exposure and direct stimulation of muscle using platinum electrodes, or *in situ* single stretch protocols create environment that may be quite different from that of a sterile strain induced mechanical insult. Although, we maximally stimulate the tibialis anterior muscle through multiple sets, our system remains intact.

There is controversy as to whether CD68 antibody is a pan macrophage marker in rats. The CD68 monoclonal antibody is used for detection of the ED1 N-glycosylated protein antigen (110kDa) which is displayed on the lysosomal membranes of immature macrophages/monocytes[28]. CD163 however, recognizes the ED2 antigen (175 kDa) that is displayed on mature, resident macrophages[28]. Unlike the bone marrow or spleen, the muscle tissue does not usually contain, or react positive for the CD68 antibody in rats unless there is an inflammatory response taking place. In control conditions muscle will stain positive for CD163 identifying resident M2 macrophages. Because we are assessing the early time points of inflammation, and the actual recruitment of monocytes to the tissue, we find these antibodies to be distinctly different. Additionally, given the plasticity of macrophages, those that react to both CD68 and CD163 are of particular interest, as this phenotype is considered anti-inflammatory; a switch would signifies the commencement of the repair and regeneration process[1].

5.4.2 Functional Force Recovery

The second aim of our study was, to determine if a single bout of massage could produce a beneficial shift in the inflammatory response, potentially resulting in the early recovery of force production associated with an attenuation of the inflammatory response. Our data analysis failed to show any significant effects of massage on torque recovery over our chosen time points of 6-, 12-, and 24 hours compared to an internal and external exercise control. Although slight trends maybe visible, a lack of power within some of our mechanical groups may have resulted in a type II error. Negative results should be interpreted with caution. Previous studies[19,20,27] have shown early functional torque recovery when massage was administered immediately following eccentric exercise in rabbits. Furthermore, a delay in massage of 24hrs completely attenuated these affects[20]. Utilization of similar methodologies would suggest that similar trends could be expected with an increase in subjects. Alternatively, these positive results were recorded at five days post injury, after four bouts of massage. There is a possibility that the time-point of 24hrs is not sufficient enough time to see potential effects, or a single bout of massage is not sufficient enough intervention to see similar results. Because previous reports have

used a rabbit model, it should be noted that differences in the immune response may differ across animal species, making comparisons difficult.

5.4.3 Future Directions

Future investigations should focus on the potential effects of various bouts of massage, its effects on the immune response, and torque production over time. When evaluating the immune response the temporal aspect is a crucial element. Investigations into the efficacy of massage should continue to be mindful to the systemic effect that takes place with massage application. Potential mechanisms regarding the systemic effects of massage should attempt to identify whether or not these effects are neurological, endocrine, or neuroendocrine related, and the extent of its action. Additionally, the evaluation of the effects of massage on the production of pro- and anti-inflammatory cytokines over time could be a powerful assessment, giving rise to potential mechanisms behind immunomodulatory effects of the microenvironment.

5.5 Conclusion

Muscle is an incredibly plastic tissue that can be stimulated by, and adapt to, a multitude of stimuli. It would appear that the inflammatory response is equally as plastic. Following injury, skeletal muscle undergoes an intricate repair and regeneration process that includes numerous critical key players associated with both: the further break down and removal of tissue; and the activation of reparative anti-inflammatory pathways. Just as with all things, balance of these systems is required for optimal outcomes. Understanding how to properly modulate, or manipulate the tissue microenvironment following injury with a manual therapy such as massage, could be a promising avenue for the treatment of skeletal muscle injury.

CHAPTER SIX: SUMMARY AND FUTURE DIRECTIONS

6.1 Summary

Although it has been shown that the mechanotransductive properties of massage have a physiological effect on the underlying skeletal muscle and its interaction with the inflammatory response, the exact mechanisms remain unknown. In the cases of non-perturbed and injured tissue, massage appears to recruit immune cells to the local area, in addition to the contralateral limb. The majority of these cells are macrophages, and because macrophages have the ability to change phenotypes based on the imposed demands of the microenvironment, it is possible that massage is influencing this transition in the early hours following application. One potential mechanism in which massage may influence these changes is by regulating the gene expression of inflammatory mediators associated with the immune response. Yet, how massage may directly effect the production of local pro- and anti-inflammatory cytokines have not been elucidated. This chapter contains a summary of the findings of this dissertation followed by some general comments.

As the title would suggest, the purpose of this body of work was to assess the immunomodulatory effects of massage in skeletal muscle. There were main three purposes for this dissertation. The first purpose was to develop a hypothetical model regarding the immunomodulatory effects of massage. The specific aim was to describe the effects of massage on the inflammatory response and local afferent nerves utilizing the principals of mechanotransduction. The second purpose was to evaluate the immunomodulatory effect of massage on non-perturbed tissue relative to applied load. The specific aim was to evaluate the response of healthy non-perturbed skeletal muscle tissue to varying magnitudes of applied load to determine if effects of massage are load dependent. Finally, the third purpose was to determine whether a single bout of massage following eccentric exercise could significantly shift the temporal inflammatory response, and whether or not a systemic effect occurs.

The effects of massage appear to be load dependent

Much of the human literature regarding massage underscored the inherent challenges of controlling its application. Often the loads are often not described, are variable across treatment sessions, or are described in general subjective terms such as 'with in a range of comfort', 'light', 'moderate', or 'as tolerated' in pressure[130,138-141]. Considering the multitude of techniques that comprise massage therapy, the variability in reported results from these studies is to be expected. The advantage of our model and the custom fabricated cyclic compressive loading device, is our ability to carefully control the load of massage application not only within a single session, but between sessions as well.

Recently, there has been one other study[97] to report a load dependent effect of massage on the recovery of function following eccentric exercise. However, to our knowledge, this work is the first to report a load dependent response of massage applied to non-perturbed tissue. In the massaged limb magnitude of load was not only related to the infiltration of immue cells, but had a significant effect on the expression level of various genes associated with the immune response. Cellular infiltration data suggested that the high magnitude of load we applied to the non-perturbed tissue (11N), likely induced a significant inflammatory response. No significant differences in cell infiltration were detected between the low (1.4) and moderate (4.5N) load. However, significant differences were determined between the low and moderate loads regarding gene expression. This suggests, based on the desired outcome, the load of massage application plays an important role. In order to manipulate, or modulate the inflammatory response, the load must not be too high in magnitude to induce further damage, but high enough to provoke changes in gene expression relative to the immune response.

Massage cross over effect appears to promote a shift in the inflammatory response

Evidence has shown in the acute stages of injury, that neutrophils (responsible for production of cytotoxic chemicals) peak at about six hours post injury. Macrophages, responsible for phagocytosis (M1) and repair (M2), become elevated in the tissue at 24 and 48 hours respectively[1,6]. Data presented in this work shows that massage application

affects the time points at which M2 macrophage peak in abundance, particularly in the early hours following injury in the non-massage limb.

M2 macrophages are resident macrophages and therefore reside in the tissue under control conditions. Their numbers do not typically begin to elevate until the M1 macrophages have cleared necrotic debris through phagocytosis. M2s then remain elevated for several days as they aid in repair and regeneration, playing major role in satellite cell activation and myoblast proliferation[3]. [1,147]. These macrophages are considered to be anti-inflammatory in nature secreting anti-inflammatory cytokines such as IL-4 and TGF-β[1]. Previously, it was unknown if massage contributed to the increase in cellular infiltration. However, we showed in the non-perturbed model, massage indeed increases the amount of cellular infiltration, M1, and M2 macrophages. An increased abundance in M2 macrophages early in the inflammatory response suggests not only that a shift in the response has taken place but that M1 macrophages recruited to the site could have undergone a phenotype switch from a pro-inflammatory profile of M1 to an anti-inflammatory profile of M2. It is possible that this event altered the local microenvironment very early in the inflammatory process and had an effect on the continued infiltration of immune cells and the overall resolution of the response.

The idea of massage providing a systemic effect has been addressed in previous literature, especially in regards to the immune system. Increases in the number of natural killer cells [118-120], and lymphocytes [118-121], in addition to their cytotoxic capacity [119,122] have been observed with massage in various models of: immune-compromised individuals (AIDS/HIV) [119,120]; subjects with cancer [118]; pre-term infants [122]; and healthy individuals [121,123]. Increases in cell number have been reported to occur as early as one day after[123] to several weeks following massage intervention.

To our knowledge this work is the first to measure the direct systemic effect of massage application over the early time course of inflammation. The increased abundance of M2 macrophages as early as 6hrs in the non-massage limb post injury reported in this work, demonstrates the early time point at which massage can have a systemic effect on acute

inflammation. According to recent work[20], the benefits of massage on the recovery of functional force are delayed if massage is not applied immediately following the eccentric exercise bout. The early time point in which we report a modulation of M2 macrophages at 6hrs, suggests that the benefits of massage are likely optimized if applied immediately following exercise induced injury.

Hypothetical model revisited

Mechanotransduction is the transformation of mechanical signal into a biological/chemical response[15]. Mechanical signal propagation from the plasma membrane to the nucleus can be transduced can be much faster then that of a ligand-receptor interaction, ~5μsec vs 5sec respectively[90]. This makes the mechanical manipulation of tissue through massage treatment an attractive modality for immunomodulation. The hypothetical model presented in chapter three hypothesized that through the properties of mechanotransduction, massage can manipulate the pro-inflammatory microenvironment of injured muscle tissue, influencing the phenotype transition of recruited macrophages. This switch in phenotype would then promote the early induction of the repair and regeneration phase through macrophage secretion of anti-inflammatory cytokines.

General cellular infiltration as measured by H&E in the exercise model, showed that even though there was less infiltration of inflammatory cells at the 6hr time point versus 12- and 24hrs, it was significantly higher then the pure control condition. This would indicate that inflammatory cells were indeed recruited to the muscle. However, unlike the other groups that experienced increases in M1 macrophages, the non-massage limb in the 6hr time point had a significant increase in M2 macrophages. Considering that the CD163 antibody marker that was used to detect M2 macrophages identifies, mature, (usually) resident, anti-inflammatory macrophages, it would appear that those macrophages recruited to the site potentially underwent a phenotype transition. Because macrophages are so sensitive to their environment it is possible that massage influenced the local microenvironment promoting this switch through the modulation of cytokine production/secretion. Although it appears that massage can influence a phenotype

change through mechanotransduction, the exact mechanisms, and the alterations of pro- and anti-inflammatory cytokine environments remain unknown.

Investigations in the field of mechanotransduction have demonstrated the highly specific signaling events that occur when muscle cells are subjected to various mechanical stimuli[17,18]. This suggests that different massage applications may result in distinct cellular events that would differ from one to the next. Additionally, considering that muscle is an isovolumetric structure, negative strains applied in one area of the muscle will likely produce positive strains distal to the application sight, resulting in different signaling events across multiple regions of the muscle. The massage application used in this work most closely resembles an effleurage technique, bi-directionally applied in distal and proximal directions parallel to muscle fiber direction. It is unknown at this time if other application techniques would produce a similar result.

6.2 Future Directions

Effects of massage on the local pro-and anti-inflammatory cytokine production. Utilizing both models of non-perturb and eccentrically damaged muscle, measuring the various pro-inflammatory cytokines following massage application over time, would provide a better representation of the local environment. Following injury an increase in pro-inflammatory cytokines such as TNF-α, and IL-β promote the recruitment of inflammatory cells, whereas the production of anti-inflammatory cytokines IL-4, and TGF-β promote repair and regeneration[1,6,8,55]. Measuring the levels of these cytokines in massaged versus contralateral and non-massaged limbs would provide insight to a potential mechanism behind the immunomodulatory effects of massage. Levels of IL-10 would be additionally valuable considering the role it plays in the transition of M1 to M2 macrophages. The hypothesis would be that massage immediately following injury, promotes the production of anti-inflammatory cytokines compared to exercise control condition.

Effects of multiple bouts of massage over time. The collective body of the mechanistic investigations into massage, utilize two models of massage application (both of which are

presented in this dissertation). Animals have either been subjected to four total bouts of massage over four days, or a single bout of massage evaluated over pre-determined time points. Investigations utilizing eccentric exercise boast early recovery of functional torque production in addition to the apparent attenuation of the inflammatory response[19,27]. It is unknown at this time whether or not a single bout, versus multiple bouts have similar effects over the same period of time. Developing a model to test single versus multiple bouts of massage over the time course of acute inflammation, would provide valuable treatment information in regards to muscle injury. The hypothesis would be that multiple bouts of massage might have additive effects capable of attenuating the inflammatory response over time versus single bout.

Effects of various massage application techniques. The custom fabricated cyclic compressive loading device has currently only been utilized to apply massage in one direction (parallel to the muscle fiber). It is unknown at this time if a perpendicular application would provide similar results. Muscle is more stiff when load is applied at an angle that is more acute to fiber orientation; as fluid moves laterally against the plane of fiber orientation it alters sheer stress[91,92]. Clinically, massage is applied in a multitude of directions. Given the specificity of cellular signaling events in relation to mechanical stimuli, characterizing the effects of massage in multiple directions of application in relation to fiber angle, would allow for the tailoring of massage treatments to match the desired outcomes. The hypothesis would be that different massage techniques promote distinct changes in the immune response.

Identification of potential mechanisms responsible for the system effects of massage. Currently the mechanism behind the changes observed in non-massaged limbs is unknown. One potential system that maybe responsible for the crossover effect that is observed in this dissertation is the nervous system. In field of neuroimmunology the 'cholinergic anti-inflammatory pathway' offers a potential hypothesis for the changes seen in the non-massaged limbs. Simulation of the afferent branch of the vagus nerve, by pro-inflammatory cytokines TNF-α, and IL-1B stimulates the release anti-inflammatory glucocorticoids processed at higher centers of the brain, in addition to modulating

cytokine production in macrophages through the efferent release of acetylcholine[148,149]. Macrophages express a nicotinic acetylcholine receptor subunit α7 that activates STAT3 pathway[150]. The STAT3 pathway inhibits the release of TNF-α[150] and initiate the production and release of anti-inflammatory IL-4, which promotes M2 polarity[151]. The connection between the vagus nerve and the mechanical manipulation of skeletal muscle tissue is unknown. However, vagus nerve stimulators and pharmaceuticals developed for vagus nerve stimulation have been utilized to successful treat individuals with autoimmune problems, including rheumatoid arthritis[148,149].

Another potential neural mechanism responsible for a crossover effect may be due to a reflex arch at the level of the spinal cord. To test this hypothesis a non-constrained exercise model should be used in which a vagotomy is performed to deduce which mechanism most likely to contributes.

Considering that the vagus nerve is part of the parasympathic nervous system, activated in times of relaxation, poses the question as to whether or not massage would be less or more effective, depending on the level of consciousness of an individual. All of the animals were anesthetized in order to perform this study. But if the vagus nerve potentially plays a role in regulating inflammation systemically, conscious recognition of relaxation may have a more profound effect.

An alternative system involved in a systemic effect could be endocrine. To assess whether or not an endocrine mechanism would be at play, serum taken from the animals following massage may be a viable source to investigate this question.

Translation of immunomodulatory effects of massage into other models of muscle damage (e.g. muscular dystrophy). Increased cytotoxicity of immune has been reported in individuals with muscular dystrophy[56]. Although mechanical disruption of the tissue results from a lack of dystrophin, it is not clear as to whether or not the break down and destruction of the tissue is a mechanical or an overzealous cellular mediated event. If massage has immumomodulatory effects there is potential that it maybe used to influence

the inflammatory response in conditions in which the response is dysregulated. Treatment options outside of pharmaceutical intervention would advantageous. It would be interesting to test the hypothesis that the extent of damage caused in muscular dystrophy is result of a dysregulated immune response.

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Curriculum Vitae
CHRISTINE WATERS BANKER

I. GENERAL INFORMATION

Born:	Hermiston, Oregon
Citizenship:	United States of America
Certificates, Licenses:	National Athletic Trainers Association Board of Certification, since 2008

II. EDUCATION

2010-Current	University of Kentucky College of Health Sciences, Rehabilitation Sciences Division of Athletic Training, Doctor of Philosophy Student
2008-2010	University of Kentucky, College of Health Sciences, Division of Athletic Training, Master of Science
2004-2008	Oregon State University, College of Health and Human Sciences, Division of Athletic Training, Bachelor of Science
2002-2004	Blue Mountain Community College, General Studies, Associate of Arts Transfer

III. PROFESSIONAL EXPERIENCES

2010-Current	Graduate Research Assistant, Department of Rehabilitation Sciences, Division of Athletic Training University of Kentucky Lexington, KY
2008-2010	Head Certified Athletic Trainer, Fayette County Middle Schools University of Kentucky Lexington, KY

IV. TEACHING ACTIVITY

University of Kentucky

Fall 2011	KHP340 Introduction to Athletic Training Primary Instructor, 34 students, 2.5 contact hours
2011-Current	AT700 Muscle Mechanics Teaching Assistant, ~20 students, 3 contact hours

Lexington Traditional Magnet School

2009-2010 Introduction to Athletic Training,
Primary Instructor, 2 Students, 1 contact hour

V. HONORS AND AWARDS

2013	Robinson Graduate Award for Research Creativity *University of Kentucky*
2012	Neuromuscular Plasticity Scholar Award *University of Florida*
2011-2013	NIH T32 Predoctoral Scholar *University of Kentucky*
2010-2011	College of Health Sciences Dean's Scholarship *University of Kentucky*
2009	Osternig Research Grant Award *National Athletic Trainers Association*
2008	Student Athletic Trainer Award *Oregon State University*
2006-2007	Athletic Training Scholarship *Oregon State University*
2006-2007	Home Economics Scholarship *Oregon State University*
2006	Deans Scholastic Achievement Award *Oregon State University*
2002-2004	Athletic Scholarship for Softball *Blue Mountain Community College*

VI. PROFESSIONAL ACTIVITY AND PUBLIC SERVICE

Membership in Professional Societies

2013-present American College of Sports Medicine
2010-present American Society of Biomechanics
2008-present Southeastern Athletic Trainers' Association
2008-present National Athletic Trainers' Association

VII. RESEARCH AND/OR CREATIVE PRODUCTIVITY

Abstracts, Scientific-refereed

Waters C, Swann W, Butterfield TA (2013) M1 Macrophages are Active as Early as Six hours Following Eccentric Exercise: A Pilot Study. National Athletic Trainers' Association (NATA) Annual Meeting & Clinical Symposia, Las Vegas, NV; June 2013 *(Accepted for poster presentation).*

Waters-Banker C, Dupont-Versteegden EE, Butterfield TA (2013)

Selective Fiber Infiltration of M1 Macrophages at Six Hours in Skeletal Muscle Following Eccentric Exercise: A Pilot Study. University of Florida Neuromuscular Plasticity Seminar, Gainsville FL, March 14, 2013 *(Accepted for poster presentation).*

Waters-Banker C, Dupont-Versteegden EE, Butterfield TA (2013). Immunomodulatory Genes in Skeletal Muscle are Responsive to Mechanical Loading Following Massage Mimetic. American College of Sports Medicine 60th Annual Conference and 4th World Congress, Indianapolis, IN, May 28-June 1, 2013. *(Accepted for thematic poster presentation).*

Moore, S, **Waters-Banker C**, Butterfield TA, Dupont-Versteegden EE (2013). ED2 Macrophage Number is Dependent upon Loading Conditions in Aged Muscle. American College of Sports Medicine 60th Annual Conference and 4th World Congress, Indianapolis, IN, May 28-June 1, 2013. *(Accepted for thematic poster presentation).*

Waters-Banker C, EE. Dupont-Versteegden, T.A Butterfield (2012) Upregulation of Autophagy through the Mechanotransductive Properties of Massage in Healthy Skeletal Muscle. Center for Clinical and Translational Science Conference, Lexington KY, March 29th, 2012 *(Accepted for poster presentation).*

Waters-Banker C, Dupont-Versteegden EE, Butterfield TA (2012) Immunomodulation and Cell Survival: The Mechanotransductive Properties of Massage. University of Florida Neuromuscular Plasticity Seminar, Gainsville FL, March 16-17, 2012 *(Accepted for poster presentation).*

Waters C, Abshire SM, Dupont-Versteegden EE, Butterfield TA. (2012) Massage Potentiates Cell Survival Mechanisms in Healthy Skeletal Muscle Through the Activation of Autophagy. National Athletic Trainers' Association (NATA) Annual Meeting & Clinical Symposia, St. Louis. MO; June 26th-29th 2012 *(Accepted for a podium presentation).*

Waters C, Butterfield TA. (2011) Effect of Cyclic Compressive Loading on ED1+ and ED2+ Macrophages in Healthy Skeletal Muscle. American Society of Biomechanics Annual Meeting, Long Beach CA; August 10-13, 2011. *(Accepted for poster presentation)*

Waters C, Butterfield TA. (2011) Alterations in Gene Expression Due to Differing Magnitudes of Cyclic Compressive Loads in Healthy Skeletal Muscle. American Society of Biomechanics Annual Meeting, Long Beach CA; August 10-13, 2011. *(Accepted for poster presentation)*

Waters CM, Abshire SM, Dupont-Versteegden EE, Butterfield TA. (2011) The Magnitude of Applied Cyclic Compressive Load Influences Inflammatory Cell Infiltration but Not Lymphangiogenesis in Healthy Skeletal Muscle. National Athletic

Trainers' Association Annual Meeting & Clinical Symposia, New Orleans, LA; June 19 to June 22, 2011. *(accepted for podium presentation, also included in Presentations)*

Abstracts, Clinical-refereed

Waters C and Butterfield TA. (2010) Spontaneous Avulsion Fracture of the Tibial Tuberosity in a Male Adolescent Football Player. National Athletic Trainers' Association 61st Annual Meeting & Clinical Symposia, Philadelphia PA June 22-25, 2010. *(Accepted for podium presentation, also included in Presentations)*

Abstracts, non-refereed

Waters-Banker C, Dupont-Versteegden EE, Butterfield TA (2012) Immunomodulation and Cell Survival: The Mechanotransductive Properties of Massage. 4th Annual Center for Muscle Biology Retreat, University of Kentucky, Lexington, KY 2012. (*Poster presentation)*

Waters C, Dupont-Versteegden EE, Butterfield TA. (2011) Alterations in Gene Expression Due to Differing Magnitudes of Cyclic Compressive Loads in Healthy Skeletal Muscle. 3rd Annual Center for Muscle Biology Retreat, University of Kentucky, Lexington, KY 2011. (*Poster presentation)*

Waters C, Dupont-Versteegden EE, Butterfield TA. (2011) The Immunomodulatory Effects of Massage. T32 Seminar presentation. Gill Heart Institute Cardiovascular Research Day. Lexington Convention Center, Lexington, KY October 21, 2011. (*Podium presentation, also included in Presentations)*

Waters C, Dupont-Versteegden EE, Butterfield TA. (2011) Alterations in Gene Expression Due to Differing Magnitudes of Cyclic Compressive Loads in Healthy Skeletal Muscle. Modeling workshop for trainees in muscle biology. University of Kentucky, Lexington, KY July 25-28th 2011. (*Poster presentation)*

Waters C, Dupont-Versteegden EE, Butterfield TA. (2011) Effect of Cyclic Compressive Loading on ED1+ and ED2+ Macrophages in Healthy Skeletal Muscle. Modeling workshop for trainees in muscle biology. University of Kentucky, Lexington, KY July 25-28th 2011. (*Poster presentation)*

Waters CM, Abshire SM, Dupont-Versteegden EE, Butterfield TA (2010) The Magnitude of Applied Cyclic Compressive Load Influences Inflammatory Cell Infiltration but Not Lymphangiogenesis in Healthy Skeletal Muscle. 2nd Annual Center for Muscle Biology Retreat, University of Kentucky, Lexington, KY 2010. (*Poster presentation)*

Oral Presentations, National

Waters C, Abshire SM, Dupont-Versteegden EE, Butterfield TA. Massage Potentiates Cell Survival Mechanisms in Healthy Skeletal Muscle Through the Activation of Autophagy. National Athletic Trainers' Association (NATA) Annual Meeting & Clinical Symposia, St. Louis. MO; June 26th-29th 2012

Waters CM, Abshire SM, Dupont-Versteegden EE, Butterfield TA. The Magnitude of Applied Cyclic Compressive Load Influences Inflammatory Cell Infiltration but Not Lymphangiogenesis in Healthy Skeletal Muscle. National Athletic Trainers' Association Annual Meeting & Clinical Symposia, New Orleans, LA; June 19 to June 22, 2011.

Waters C and Butterfield TA. Spontaneous Avulsion Fracture of the Tibial Tuberosity in a Male Adolescent Football Player. National Athletic Trainers' Association 61st Annual Meeting & Clinical Symposia, Philadelphia PA June 22-25, 2010.

Oral Presentations, Local

Waters C, Dupont-Versteegden EE, Butterfield TA. The Immunomodulatory Effects of Massage. T32 Seminar presentation. Gill Heart Institute Cardiovascular Research Day. Lexington Convention Center, Lexington, KY. October 21, 2011.

Waters C. Don't Rub Me the Wrong Way: Magnitude Dependent Inflammatory Response to "Massage" in Muscle. Department of Physiology Muscle Forum. University of Kentucky, Lexington, KY. May 20, 2011.

Waters C, and Butterfield TA. Knee Injury in an Adolescent Male Football Athlete. 12th Annual Wildcat Symposium. University of Kentucky Orthopaedics & Sports Medicine, Lexington, KY. May 15th, 2010.

Presentations, Invited

Waters-Banker C. Don't Rub Me the Wrong Way- What's the Message on Massage? 15th Annual UK Sports Medicine Symposium, Lexington, KY May 17, 2013 (*Three 30minute sessions).*

Publications

Waters-Banker C, Butterfield TA, Dupont-Versteegden EE (2013). Immunomodulatory Effects of Massage Applied to Non-Perturbed Skeletal Muscle in Rats. (***In revison***)

Waters-Banker C, Dupont-Versteegden EE, Kitzman PH, Butterfield TA (2013). Investigating the Mechanisms of Massage Efficacy: the Role of Mechanical Immunomodulation. *J Athl Train* (***In press***)

Dupont-Versteegden EE, **Waters C**. Muscle Atrophy without GSK-3beta. Focus on "Glycogen synthase kinase-3beta is required for the induction of skeletal muscle atrophy." *Am J Physiol Cell Physiol*. 2011; 301(5):C980

Publications in Progress

Waters-Banker C, Dupont-Versteegden EE, Butterfield TA. Crossover Effect of Massage Following Eccentric Exercise Influences Abundance of Macrophage Populations at Six Hours. (To be submitted to *The Journal of Physiology*)

Waters-Banker C, McKeon JM, Bush HM. Technical Note: Survival Analysis. (To be submitted to *Journal of Athletic Training*).

Waters-Banker C, Bush HM. Technical Note: Hazard Ratios. (To be submitted to *Journal of Athletic Training).*

Waters-Banker C, Bush HM. Technical Note: Repeated Measures. (To be submitted to *Journal of Athletic Training*).

VIII. GRANT ACTIVITY

Submitted-Extramural

Approved and Funded-Extramural

National Athletic Trainers' Association Research Foundation Osternig Master's Grant

Title: Effects of Massage on Lymphangiogenesis and Inflammation
Investigators: **Waters C**, and Butterfield TA
Status: Funded 7/16/2009 thru 7/15/10
Amount: $984.00 (3048106834)
Roles: Waters C-Mentee, Butterfield TA-Mentor

Approved and Funded-Intramural

Submitted and Not Funded-Extramural

National Institutes of Health/NCCAM (F31) –Scored: 52

Title: Quantification of the Load Dependent Immunomodulatory Effects of Massage
Investigators: **Waters CM**, and Butterfield TA
Status: Submitted 4/08/2011
Total Amount: $144,000.00
Roles: Waters CM-Mentee, Butterfield TA-Mentor

National Institutes of Health/NCCAM (F31) -Resubmission

Title: Quantification of the Load Dependent Immunomodulatory Effects of Massage
Investigators: **Waters CM**, and Butterfield TA
Status: Submitted 8/08/2011
Total Amount: $144,000.00
Roles: Waters CM-Mentee, Butterfield TA-Mentor