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Acute Pro-inflammatory Immune Response Following Different Resistance Exercise Protocols in Trained Men

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ACUTE PRO-INFLAMMATORY IMMUNE RESPONSE FOLLOWING DIFFERENT
RESISTANCE EXERCISE PROTOCOLS IN TRAINED MEN

by

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ABSTRACT

The successful regeneration of muscle tissue is dependent upon the infiltration of phagocytic $CD14^{++}CD16^-$ monocytes that support the proliferation and differentiation of myogenic precursor cells. Physiologically, the magnitude of the cellular response following resistance exercise is dictated by the level of receptor expression on the plasma membrane of the monocyte, as well as the secretion of their cognate ligands from tissue resident cells. However, it remains unclear whether the innate pro-inflammatory immune response varies with different resistance training protocols, and how it may impact recovery and the muscle remodeling process. Therefore, the purpose of this investigation was to examine temporal changes in the expression of chemotactic and adhesion receptors following an acute bout of high-volume, moderate-intensity (VOL) versus high-intensity, low-volume (HVY) lower-body resistance exercise in experienced, resistance trained men. Changes in receptor expression were assessed in conjunction with plasma concentrations of MCP-1, TNFα, and cortisol. Ten resistance-trained men (90.1 ± 11.3 kg; 176.0 ± 4.9 cm; 24.7 ± 3.4 yrs; $14.1 \pm 6.1\%$ body fat) performed each resistance exercise protocol in a random, counterbalanced order. Blood samples were obtained at baseline (BL), immediately (IP), 30 minutes (30P), 1 hour (1H), 2 hours (2H), and 5 hours (5H) post-exercise. Analysis of target receptor expression on $CD14^{++}CD16^-$ monocytes was completed at BL, IP, 1H, 2H and 5H time points via flow cytometric analysis. Plasma concentrations of myoglobin, and LDH AUC were significantly greater following HVY compared to VOL ($p = 0.003$ and $p = 0.010$ respectively). Changes in plasma TNFα, MCP-1, and expression of CCR2, CD11b, and GCR on $CD14^{++}CD16^-$ monocytes were similar following HVY and VOL. When collapsed across groups, TNFα was significantly increased at IP, 30P, 1H and 2H post-exercise

(p = 0.001 – 0.004), while MCP-1 was significantly elevated at all post-exercise time points (p = 0.002 – 0.033). CCR2 expression was significantly lower at IP, 1H, 2H and 5H post-exercise (p = 0.020 – 0.040). In contrast, CD11b receptor expression was significantly greater at 1H relative to BL (p = 0.001), while GCR expression was not significantly different from baseline at any time point. As expected, plasma cortisol concentrations were significantly higher following VOL compared to HVY (p = 0.001), although this did not appear to be related to changes in receptor expression. Plasma testosterone concentrations and TNFr1 receptor expression did not appear to be affected by resistance exercise. Our results do not support a role for cortisol in the modulation of CCR2 receptors *in vivo*, while the degree of muscle damage does not appear to influence plasma concentrations of TNFα, or MCP-1. It is therefore likely that both HVY and VOL protocols constitute an exercise stimulus that is sufficient enough to promote a robust pro-inflammatory response, which is similar in timing and magnitude.

"Perform whatsoever it is that thou art about, with true and unfeigned gravity, natural affection, freedom and justice"

– Marcus Aurelius

To my parents Graham and Michele, for supporting me in all my endeavors and teaching me that anything is possible.

To my brothers Mathew and Lee, for being great examples of how to be a man. I look up to you both.

To my girlfriend Amber, for your patience and understanding during this process, I love you!

To Todd and Tiffany Griffin, my closest friends, I'll see you on the porch in 50 years.

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LIST OF ACRONYMS/ABBREVIATIONS

1H	One hour Post-exercise
1RM	1-Repetition Maximum
2H	Two Hours Post-Exercise
30P	30 Minutes Post-exercise
5H	Five Hours Post-exercise
Akt	Protein Kinase B
ANOVA	Analysis of Variance
APC	Allophycocyanin
AUC	Area Under the Curve
BL	Baseline
CCR2	C-C Chemokine Receptor 2
CCR2$^{-/-}$	C-C Chemokine Receptor 2 Knockout
CD115	Colony Stimulating Factor 1 Receptor
CD11a	Lymphocyte function-associated antigen 1
CD11b	Integrin αM
CD14	Cluster of Differentiation 14
CD16	Cluster of Differentiation 16
CD18	Integrin β_2
CD62L	Cluster of Differentiation 62L (L-Selectin)
cKIT	Mast/Stem Cell Growth factor Receptor
CNS	Central Nervous System
CTX	Cardiotoxin
CX$_3$CR1	CX3 Chemokine Receptor 1

d	Cohen's *d* Effect Size
DC	Dendritic Cell
DNA	Deoxyribonucleic Acid
ED1	Ectodermal Dysplasia Antibody 1
ED2	Ectodermal Dysplasia Antibody 2
ED3	Ectodermal Dysplasia Antibody 3; Sialoadhesin
ELISA	Enzyme-linked Immunosorbent Assay
ERK1/2	Extracellular-signal-regulated Kinase 1 and 2
F4/80	EGF-like Module-containing Mucin-like Hormone Receptor-like 1
FBS	Fetal Bovine Serum
Flt3	FMS-like Tyrosine Kinase 3
FOXO	Foxhead Box O
FSC-A	Forward Scatter Area
FSC-H	Forward Scatter Height
GCR	Glucocorticoid Receptor
GTP	Guanosine-5'-Triphosphate
HPL	Human Performance Laboratory
HVY	High-Intensity, Low-Volume Protocol
ICAM-1	Intracellular Adhesion Molecule 1
IGF-1	Insulin-like Growth Factor 1
IgG	Immunoflobulin G
IP	Immediately Post-exercise
K_2EDTA	Ethylenediaminetetraacetic Acid
LDH	Lactate Dehydrogenase
LFA-1	Lymphocyte-function Associated Antigen 1

Lin1	Lineage Associated Antigen 1
LSD	Least Significant Difference
Ly6C	Lymphocyte Antigen 6C
M1	Pro-inflammatory Macrophage
M2	Anti-inflammatory Macrophage
MCP-1	Monocyte Chemotactic Protein 1
MCP-1$^{-/-}$	MCP-1 Knockout
MCP-3	Monocyte Chemotactic Protein 3
MDP	Macrophage and DC Precursor
MPC	Myogenic Precursor Cell
MRF	Myogenic Regulatory Factor
mRNA	Messenger Ribonucleic Acid
n^2_p	Partial Eta Squared
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate-Buffered Saline
PE	Phycoerythrin
RM-1	Anti-HepatitisB Surface Antigen Antibody
ROS	Reactive Oxygen Species
RU486	Anti-glucocorticosteroid
SD	Standard Deviation
Th1	T Helper Cell Pro-inflammatory Cytokine
Th2	T Helper Cell Anti-inflammatory Cytokine
TNFr1	Tumor Necrosis Factor Receptor 1
TNFr2	Tumor Necrosis Factor Receptor 2
TNFα	Tumor Necrosis Factor Alpha

VO$_{2max}$ Maximal Oxygen Uptake

VOL   High-Volume, Moderate-Intensity Protocol

W3/13   Sialophorin

CHAPTER I: INTRODUCTION

Changes in acute program variables (i.e., intensity, volume and rest) during resistance training may result in a different physiological stimulus to the muscle (Bogdanis, 2012). If the stimulus is of significant magnitude, often at a level that is greater than the muscle is accustomed to, significant damage to the recruited musculature may be observed (Clarkson & Hubal, 2002). Damaged myofibers subsequently undergo either apoptotic or necrotic cell death (Wynn & Barron, 2010), leading to the production of cellular debris. Recent evidence suggests that the presence of cellular debris may interfere with muscle regenerative processes, acting as a physical barrier that prevents myoblasts from efficiently contacting and fusing with existing myofibers (H. Lu, Huang, Ransohoff, & Zhou, 2011). This has been reported to result in delayed and impaired muscle regenation, characterized by increased fibrosis (Shen, Li, Tang, Cummins, & Huard, 2005), and fat infiltration (Summan et al., 2006; Wang et al., 2014). Consequently, the removal of cellular debris following myotrauma appears to be important for successful muscle regeneration.

The clearance of cellular debris is accomplished by specialized phagocytic macrophage cells that possess specific receptors dedicated to the envelopment of pathogens and opsonized tissue (Aderem & Underhill, 1999). Phagocytic macrophages are derived from circulating $CD14^{++}CD16^-$ monocytes, which are recruited to the site of muscle damage (Soehnlein & Lindbom, 2010; Wermuth & Jiminez, 2015). Upon entry into the tissue, cytokines and chemokines produced by local innate immune cells drive monocyte differentiation towards the pro-inflammatory macrophage (M1) phenotype (Ginhoux & Jung, 2014; Wermuth & Jiminez, 2015), which support the clearance of opsonized tissue and the proliferation of myogenic precursor cells (MPC's) (Arnold et al., 2007; Saclier et al., 2013). Abrogation of C-C chemokine

receptor 2 (CCR2) and/or endothelial adhesion receptor CD11b on $CD14^{++}CD16^{-}$ monocytes has been shown to result in an attenuated macrophage accumulation, and subsequent prevention of muscle regeneration (Arakawa et al., 2010; Arnold et al., 2007; Brodmerkel et al., 2005; Chazaud et al., 2003; Contreras-Shannon et al., 2007; Mesri, Plescia, & Altieri, 1998; Ochoa et al., 2007; H. Rosen & Gordon, 1987; Schenkel, Mamdouh, & Muller, 2004; Tsou et al., 2007; Volpe et al., 2012; Warren et al., 2004; Willenborg et al., 2012). Therefore, the successful infiltration of monocytes into damaged tissue is paramount for optimal tissue remodeling.

$CD14^{++}CD16^{-}$ monocytes are reported to possess gene enrichment in areas that make them particularly receptive to environmental stimuli. These stimuli include bacterial components, toxins, drugs, hypoxia, nutrient levels, and hormones (Wong et al., 2011). Resistance exercise alone is a potent stimulus for acute increases in the concentrations of circulating hormones (Smilios, Pilianidis, Karamouzis, & Tokmakidis, 2003). Perturbations to the systemic hormone profile may subsequently dictate the timing and magnitude of the inflammatory response (Kiess & Belohradsky, 1986; Walsh et al., 2011), which in turn may lead to altered tissue adaptation. Glucocorticoids in particular have been implicated with innate immune-regulation, potentiating an up-regulation in the expression of chemotactic receptor CCR2 on human monocytes *in vitro* (Okutsu, Suzuki, Ishijima, Peake, & Higuchi, 2008; Penton-Rol et al., 1999; Pettersson et al., 2005). High-volume resistance exercise protocols may stimulate a differential pattern of receptor expression, since these protocols are often reported to result in greater circulating cortisol concentrations compared to low-volume resistance exercise (Ahtiainen, Pakarinen, Kraemer, & Hakkinen, 2003; Kraemer & Ratamess, 2005). Nevertheless, a comparison of monocyte cell surface protein expression following different resistance training protocols, to our knowledge, has not yet been conducted.

The presence of a number of other ligands within the circulation may also regulate the expression of specific cell surface receptors. For example, both monocyte chemotactic protein – 1 (MCP-1) and tumor necrosis factor alpha (TNFα) have been shown to modulate both CCR2 and CD11b receptor expression on leukocytes (Gamble, Harlan, Klebanoff, & Vadas, 1985; Montecucco et al., 2008; K. Takahashi et al., 2003; Vaddi & Newton, 1994; Weber et al., 1999). However, the effect of different resistance exercise protocols on circulating MCP-1 and TNFα levels is not known. It remains unclear whether the innate pro-inflammatory immune response varies with different resistance training protocols, and how it may impact recovery and the muscle remodeling process. Therefore, the purpose of this investigation was to examine temporal changes in the expression of chemotactic and adhesion receptors following an acute bout of high-volume, moderate-intensity (VOL) versus high-intensity, low-volume (HVY) lower-body resistance exercise in experienced, resistance trained men. Specifically, the expression of tumor necrosis factor receptor-1 (TNFr1), C-C chemokine 2 (CCR2), cluster of differentiation (CD)11b, and glucocorticoid receptor (GCR) were assessed. Changes in receptor expression were assessed in conjunction with changes to plasma concentrations of MCP-1, TNFα, and cortisol.

CHAPTER II: LITERATURE REVIEW

Skeletal muscle demonstrates a profound capacity to adapt to an array of physiological demands (Hawke & Garry, 2001). Episodic bouts of muscle contraction are potent stimuli in this regard; which under optimal conditions, leads to the remodeling and functional adaptation of skeletal muscle tissue in response to mechanical stress (Egan & Zierath, 2013; Hawke & Garry, 2001). Manipulation of the mechanical stimulus has been suggested to impart varying degrees of skeletal muscle adaptation (Kraemer & Ratamess, 2005). This stimulus is primarily modulated through changes to acute program variables such as exercise intensity, volume and rest intervals, which evoke distinct mechanical and biochemical responses that subsequently lead to contrasting contractile phenotypes (Hornberger, 2011).

Traditionally, resistance training paradigms are divided into either high-intensity or high-volume protocols. High-intensity protocols typically involve heavy loads [($\geq 85\%$ repetition maximum (1RM)], low volumes (2-6 sets; ≤ 6 repetitions), and longer rest intervals (3-5 minutes), while high-volume protocols typically involve more moderate intensities [$< 85\%$ 1RM)], higher volumes (3-6 sets; 8-12 repetitions), and shorter rest intervals (30 – 90 seconds) (Bird, Tarpenning, & Marino, 2005; Tan, 1999). High-intensity, low-volume protocols are thought to result in greater strength gains, which may be related to the recruitment of high-threshold motor units inherent with heavier loading (Kraemer & Ratamess, 2004; Sale, 1987). In contrast; high-volume, moderate-intensity protocols are generally thought to stimulate muscle hypertrophy, which may be mediated, in part, by concomitant elevations in systemic hormones that are thought to augment the anabolic response (Crewther, Cronin, Keogh, & Cook, 2008; McCaulley et al., 2009; Smilios et al., 2003). Notwithstanding, the optimal configuration of

program variables for the purpose of maximizing strength and/or muscle growth remain unclear (Adams & Bamman, 2012). However, it is likely that skeletal muscle regeneration/adaptation depends upon the finely coordinated interactions of a number of distinct biological systems that work together for the resolution of tissue homeostasis (Kharraz, Guerra, Mann, Serrano, & Munoz-Canoves, 2013).

Muscle Regeneration

Adult skeletal muscle fibers are terminally differentiated cells (Hawke & Garry, 2001). Because the nuclei within these fibers are post-mitotic, myogenesis depends primarily upon a population of satellite cells that are crucial for muscle growth and repair (Bondesen, Mills, Kegley, & Pavlath, 2004). Under conditions of homeostasis, satellite cells remain in a non-proliferative quiescent state, and are physically distinct from adult myofibers due to their confinement to indentations between the basal lamina and sarcolemma of the muscle fiber (Hawke & Garry, 2001). However, in response to myotrauma, quiescent satellite cells become activated (at which stage they are refereed to as MPC's), and begin to proliferate. While a small subset of activated satellite cells subsequently return to quiescence in a process of self-renewal (Brack & Rando, 2012; Charge & Rudnicki, 2004), most satellite cells will differentiate and fuse to damaged myofibers for the purpose of restoring tissue integrity and function (Martin & Lewis, 2012). The satellite response has been recently associated with the extent of training induced muscle hypertrophy (Bellamy et al., 2014). This process, decribed in detail by Charge and colleagues (2004), is illustrated in Figure 1. Following damage to the myofiber, activated quiescent satellite cells begin to proliferate, allowing for expansion of the myogenic cell

population (A). At the molecular level, activation of MPC's in response to myotrauma is mediated by the up-regulation of a number of muscle regulatory factors (MRF's), which lead to the commitment of satellite cells to the myogenic lineage (Sabourin & Rudnicki, 2000). Following the proliferative phase, committed satellite cells (myoblasts) travel to damaged myofibers in response to chemical stimuli (chemotaxis) (B), where they fuse to damaged myofibers and become terminally differentiated mononucleated myocytes (Hindi, Tajrishi, & Kumar, 2013) (C). During fusion, myoblasts provide new myonuclei to the existing myofibers, which grow to resemble the original myofiber. As regeneration progresses, donated myonuclei coalesce towards the center of the regenerated myofiber (D) resulting in the characteristic centronucleated appearance (Bosurgi, Manfredi, & Rovere-Querini, 2011). As regeneration is completed, these myonuclei migrate to the periphery of the fiber and begin to resemble normal muscle fibers (E).
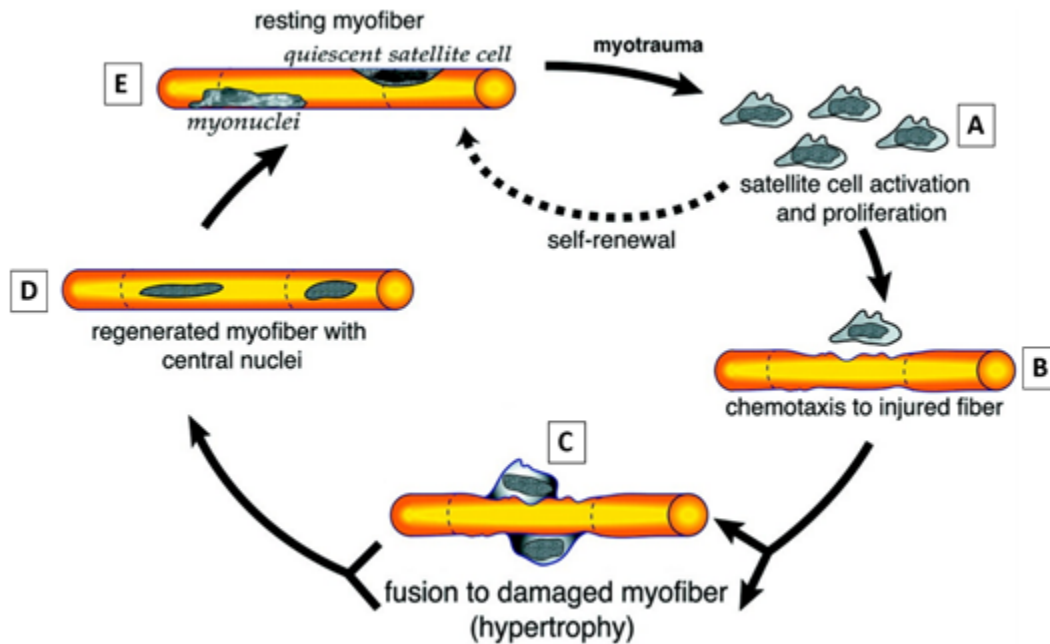
Figure 1. Satellite cell response to myotrauma.
Adapted from Myogenic satellite cells: physiology to molecular biology. Hawke and Garry (2001). Included with permission.

While it is widely accepted that the satellite cell response is fundamental for the reconstitution of muscle integrity following myotrauma, skeletal muscle regeneration is a functionally complex phenomenom. In addition to the role of MPC's, many other cell types play a role in optimizing the regenerative processes. These cells include endothelial cells, interstitial cells, mesenchymal progenitors, fibro/adipogenic progenitors, and inflammatory cells (Ceafalan, Popescu, & Hinescu, 2014). Alongside satellite cells, inflammatory cells appear to be essential for successful muscle regeneration (Kharraz et al., 2013). In particular, it is widely recognized that macrophages execute a critical role in muscle repair (Brunelli & Rovere-Querini, 2008; Robertson, Maley, Grounds, & Papadimitriou, 1993; Saclier et al., 2013; Tidball & Wehling-Henricks, 2007; Wang et al., 2014).

<u>Macrophages and Muscle Repair</u>

Resistance exercise results in damage to parenchymal cells (myofibers), which subsequently undergo either apoptotic or necrotic cell death (Wynn & Barron, 2010). The clearance of these apoptotic/necrotic cells (cellular debris) is accomplished by specialized phagocytic macrophage cells that possess specific receptors dedicated to the envelopment of pathogens and cellular debris (Aderem & Underhill, 1999). The removal of cellular debris following tissue damage appears to be a critical event that directly mediates muscle regeneration (Bosurgi et al., 2011). Recent evidence suggests that the presence of necrotic fibers may interfere with muscle regenerative processes, acting as a physical barrier that prevents myoblasts from efficiently contacting and fusing with existing myofibers (H. Lu et al., 2011). Consistent with this, impaired muscle regeneration is reported to be prevalent following *in vitro* macrophage depletion (Brunelli & Rovere-Querini, 2008)**,** indicating that the macrophage-mediated removal of cellular debris is likely critical for optimal tissue regeneration. However, upon the cessation of phagocytosis, macrophages remain present in regenerating areas, often in high numbers, and are tightly associated with MPC's and young regenerating myofibers (Saclier, Cuvellier, Magnan, Mounier, & Chazaud, 2013). Additionally, depletion of intramuscular macrophages following complete phagocytosis has been shown to result in reduced muscle fiber size during recovery from myotrauma (Arnold et al., 2007). This indicates that macrophages may have the ability to promote muscle regeneration independent of phagocytic function.

In vitro studies suggest that macrophages directly support MPC growth via the secretion of soluble mitogenic factors (Chazaud et al., 2003; Merly, Lescaudron, Rouaud, Crossin, & Gardahaut, 1999). In particular, macrophage derived insulin-like growth factoe-1 (IGF-1)

appears to have a role in mediating tissue repair. IGF-1 exerts its function via the phosphorylation of Akt which leads to MPC proliferation and myoblast differentiation (Engert, Berglund, & Rosenthal, 1996; Schiaffino & Mammucari, 2011). Following myotrauma, macrophage accumulation has been shown to correlate with an up-regulation of both mRNA and protein expression of IGF-1 (Heinemeier et al., 2009; H. Lu et al., 2011). Additionally, it has been reported that impaired muscle regneration following macrophage depletion is associated with a concomitant decrease in IGF-1 mRNA within the muscle (Summan et al., 2006). Further, the recently described protective effect of macrophages against myotube atrophy (N. A. Dumont & Frenette, 2013) is abolished following administration of an anti-IGF-1 antibody treatment (N. Dumont & Frenette, 2010). Therefore, it is reasonable to suggest that the role of macrophages in skeletal muscle regeneration may be mediated, at least in part, by the production of mitogenic factors including IGF-1.

Recent studies have established that macrophages exert different effects on MPC's, dependent upon their polarization to either the pro-inflammatory (M1) or anti-inflammatory (M2) phenotype. M1 macrophages appear to stimulate MPC proliferation and inhibit their fusion (Arnold et al., 2007; Saclier et al., 2013), while M2 macrophages appear to stimulate myogenesis by promoting the terminal differentiation of myoblasts and the formation of large myotubes (Saclier et al., 2013). M2 macrophages are likely derived from M1 macrophages and thus represent a latter stage of inflammatory macrophage differentiation. It has been posited that the removal of cellular debris may serve as a key signal prompting the functional shift in macrophage phenotype (Arnold et al., 2007; Rigamonti, Zordan, Sciorati, Rovere-Querini, & Brunelli, 2014; Tidball & Villalta, 2010).

Macrophages have been associated with the "rescuing" of myotubes from apoptosis following skeletal muscle injury (Chazaud et al., 2003; Lesault et al., 2012; Sonnet et al., 2006). The addition of macrophages to primary MPC cultures *in vitro* appears to lead to an inhibition of spontaneous myoblast and myotube apoptosis that coincides with the enhanced expression of anti-apoptotic proteins and activation/phosphorylation of ERK1/2 and Akt pathways (Sonnet et al., 2006). It is possible that macrophage derived IGF-1 may mediate these anti-apoptotic effects, since IGF-1 is known to facilitate the inhibition of the foxhead box O (FoxO) apoptotic signaling (Stitt et al., 2004; Zhang, Tang, Hadden, & Rishi, 2011). FoxO transcription factors mediate cell cycle arrest, DNA repair and apoptosis (Lam, Francis, & Petkovic, 2006). However; to our knowledge, the role of macrophage derived IGF-1 in mediating FoxO signalling has not been addressed. Nevertheless, macrophages may exert their anti-apoptotic fuinction through mechanisms independent of IGF-1 signalling. DNA microarray analyses indicates that MPCs constitutively express four cell-to-cell molecular adhesion receptors, all of which are functionally active and responsive to counterligands expressed by human macrophages. Blockade of any one of these adhesion receptors results in the inhibition of the beneficial effect of macrophages on MPC survival. Therefore, the delivery of anti-apoptotic signals appears to be mediated by cell-to-cell contact between macrophages and MPCs (Chazaud et al., 2003; Sonnet et al., 2006). These receptor systems are more strongly expressed by myotubes (multinucleated differentiated myogenic cells) that are more protected from apoptosis compared with undifferentiated myoblasts, suggesting that macrophages could help protect these cells until they establish a final association with the extracellular matrix (Chazaud et al., 2009).

Current evidence suggests that macrophages exert their effect in three ways: 1. via the phagocytosis of opsonized cellular debris; 2. via production of soluble factors that globally

stimulate MPC proliferation and differentiation; and 3. via delivery of anti-apoptotic signals through direct cell contact with MPCs (Aderem & Underhill, 1999; Chazaud et al., 2003; Sonnet et al., 2006). The influence of macrophages in the resolution of tissue damage is depicted in Figure 2. Briefly, the tibialis anterior and quadriceps muscles of two month old C57BL/6 mice were injected with cardiotoxin (CTX) to induce muscle damage. Mice were subsequently treated, or not, with clodronate-containing liposomes to deplete macrophages, and were subsequently sacrificed 15-days following CTX injection. Histochemical analysis of the harvested muscle tissue was performed at day 15. Panel A depicts the healthy muscle of untreated control mice. Myonuclei (dyed in blue) are confined to the periphery of the myofibers, indicating a healthy mature muscle cell. Panel B depicts the muscle tissue of macrophage-competent mice 15-days post-CTX injection. Centronucleated fibers are evident throughout the section indicating effective and almost complete regeneration. Panel C depicts the muscle tissue of marophage depleted mice 15-days post-CTX injection. Muscle regeneration is significantly impaired and degenerative fibers and cell remnants persist.
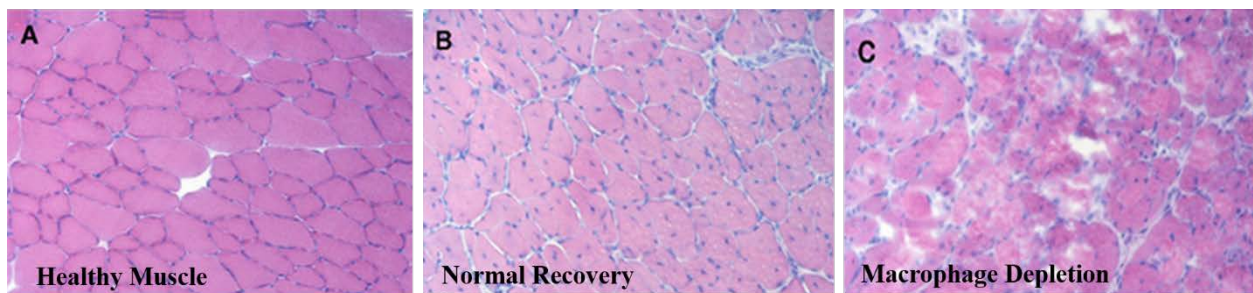
Figure 2. Macrophages and tissue regeneration.
Adapted from *Macrophages in injured skeletal muscle: A perpetuum mobile causing and limiting fibrosis, prompting or restricting resolution and regeneration.* Bosurgi, Manfredi and Rovere-Querini (2011). Included with permission. Changes have been made to the descriptions of each image.

Skeletal muscle has been shown to contain a large population of resident macrophages (Honda, Kimura, & Rostami, 1990). However, macrophages are also known to originate from circulating monocytes (Kumar & Jack, 2006). In light of this, previous studies have attempted to examine the role of both resident and monocyte derived macrophages in muscle regenerative processes following acute skeletal muscle injury (McLennan, 1993; McLennan, 1996). The origin of macrophages involved in the removal of degenerating muscle fibers has previously been investigated *in vivo* using ectodermal dysplasia antibodies ED1 (CD68), ED2 (CD163), and ED3 (CD169), as well as RM-1 and W3/13 (CD43) monoclonal antibodies (Honda et al., 1990; McLennan, 1993; McLennan, 1996). These antibodies are specific to mononuclear phagocyte system (MPS) antigens, and thus can be utilized to differentiate and examine macrophage phenotype in various tissues. The ED1 antibody is expressed by monocytes and monocyte derived pro-inflammatory macrophages. ED2 and ED3 antibodies bind to certain subsets of resident macrophages, including those in skeletal muscle, without binding to pro-inflammatory macrophages, dendritic cells, or their monocytes precursors (McLennan, 1993). RM-1 antibodies are also specific to monocytes and a sub-population of resident macrophages, while W3/13 antibodies are specific to neutrophils. Staining of degenerating muscle fibers with monoclonal antibody antigens specific to these macrophage and neutrophil phenotypes have enabled the identification of cell subtypes involved with muscle regeneration using immunohistochemistry. Additionally, the spatial and temporal distributions of these cells following acute muscle injury have been characterized (McLennan, 1993; McLennan, 1996).

Observational studies indicate that different subtypes of hemopoietic cells are attracted to a lesion by distinct signals, and have differing functions in the process of muscle regeneration. According to these observations, W3/13 neutrophils are the first cells to penetrate and leave damaged tissue (Fielding et al., 1993; McLennan, 1996). Neutrophils are apparent in the epimysium overlying tissue damage from freeze lesioning at 1 hour post-injury, and are widely distributed throughout damaged tissue 7 hours following freeze lesioning of the tibialis anterior muscle in Wistar rats (McLennan, 1996). However, these cells are rarely observed to penetrate into muscle fibers during the regenerative process (McLennan, 1996). While their exact function is not clearly defined, recent evidence suggests that the role of neutrophils is to initiate the repair process that becomes subsequently managed by macrophages (Butterfield, Best, & Merrick, 2006). This may be achieved through the generation of soluble factors such as proteases and reactive oxygen species (ROS) (Pizza, McLoughlin, McGregor, Calomeni, & Gunning, 2001; G. M. Rosen, Pou, Ramos, Cohen, & Britigan, 1995). These factors appear to contribute to secondary tissue damage (Kharraz et al., 2013; Nguyen, Lusis, & Tidball, 2005), which may serve to modify the intramuscular environment in preparation for the phagocytosis of tissue debris by macrophages. *In vivo* analysis in human subjects confirm the temporal appearance of neutrophils observed in the murine model. Following 45 minutes of downhill running in untrained men, Fielding and colleagues (1993) reported a significant infiltration of neutrophils to the exercised tissue within an hour of exercise cessation.

ED2[+] and ED3^{+} macrophages, although abundant throughout healthy muscle tissue, do not appear to be present within degenerating muscle fibers (McLennan, 1993; McLennan, 1996). ED2^{+} macrophages reportedly accumulate in the epimysium and perimysium surrounding tissue lesions, but do not penetrate into the lesion until extensive phagocytosis has already occurred

(usually 1 or 2 days). Pursuant to penetration, ED2$^+$ cells are concentrated in the regenerating

connective tissues and empty remnants of phagocytized fibers, but are rarely observed to invade

necrotic tissue, even when immediately adjacent to it (McLennan, 1993; McLennan, 1996). This

suggests that this type of macrophage has a specialized function which is unrelated to removal of

damaged tissue.

Undamaged muscle tissue appears to be essentially devoid of ED1$^+$ macrophages.

However; following damage, a small number of ED1$^+$ cells (monocyte derived cells) appear

within an hour of tissue lesioning, with subsequent cellular infiltration of these cells becoming

apparent within 3 hours (McLennan, 1996). ED1$^+$ macrophages subsequently predominate

during the initial response to skeletal muscle damage, taking charge in the clearance of

opsonized cellular debris (Brigitte et al., 2010). Their absence from healthy tissue suggests that

ED1$^+$ pro-inflammatory macrophages are derived from monocytes that enter the tissue from

adjacent arterioles in response to muscle damage. This notion is supported by more recent

evidence that suggests that MPCs depend on the support of stromal cells (supportive cells),

including monocyte/macrophages, to develop their myogenic program (Seale, Asakura, &

Rudnicki, 2001; Spradling, Drummond-Barbosa, & Kai, 2001). Consistent with this, all

phagocytic macrophages are reported to be ED1$^+$/ED2$^-$/ED3$^-$. MPCs *in vitro*, appear to

selectively and specifically recruit monocytes through the endothelium in a dose-dependent

manner (Chazaud et al., 2003). This recruitment varies according to myogenic maturation,

whereby chemotactic activity progressively declines during the later stages of myogenic

differentiation, after peaking immediately following MPC release from quiescence. Furthermore,

the depletion of circulating monocytes in mice at the time of muscle injury has been shown to

completely prevent muscle regeneration (Arnold et al., 2007), reinforcing the notion that

circulating blood monocytes supply the peripheral tissues with macrophage precursors (Serbina, Jia, Hohl, & Pamer, 2008).

Human Monocyte Subsets

Monocytes constitute approximately 10% of the total circulating leukocyte population in humans (Italiani & Boraschi, 2014). Peripheral circulating monocytes exhibit morphological heterogeneity, manifested through variations in size, granularity and nuclear morphology (Gordon & Taylor, 2005; Strauss-Ayali, Conrad, & Mosser, 2007; Yona & Jung, 2010). However, in terms of function, peripheral blood monocytes in humans are characterized based upon their differential expression of antigenic markers, rather than their morphological characteristics. Derived in the bone marrow from a common $Lin1^-cKit^{hi}CD115^+CX_3CR1^+Flt3^+$ progenitor cell (macrophage and DC precursor; MDP) (Fogg et al., 2006), circulating human monocytes were initially identified based upon their high expression of CD14 antigen (Griffin, Ritz, Nadler, & Schlossman, 1981). Subsequent research has since determined that a number of sub-populations of human peripheral blood monocytes exist, which are distinguishable based upon the differential expression of CD14 and CD16 antigens. Until recently, monocyte heterogeneity was mostly understood based upon the categorization of 2 subpopulations; the $CD16^-$ classical monocyte, and the non-classical $CD16^+$ monocyte. However, a panel of leading experts in monocyte biology recently proposed consensus nomenclature for the re-classification of human monocytes into three distinct subsets, which has since been approved by the International Union of Immunological Societies (L. Ziegler-Heitbrock et al., 2010). These subpopulations were subsequently redefined as classical ($CD14^{++}CD16^-$), non-classical

(CD14$^+$CD16^{++}) and intermediate (CD14^{++}CD16$^+$) monocytes. However, this characterization is still in its infancy. Consequently, for the purposes of this review, monocyte heterogeneity will be discussed in relation to classical (CD14^{++}CD16$^-$) and non-classical (CD14$^+$CD16^{++}) monocyte only.

To date, the functional characteristics of human monocyte subsets have been primarily investigated through the examination of murine models as a surrogate for the study of human monocyte behavior *in vivo* (Geissmann, Jung, & Littman, 2003; Gordon & Taylor, 2005; L. Ziegler-Heitbrock et al., 2010). Although monocyte subsets in humans and mice are not precisely analogous, their differentiation and contribution to immune defense appear to be similar (Belge et al., 2002; Ingersoll et al., 2010; Shi & Pamer, 2011). Consequently, the murine model of *in vivo* monocyte behavior has provided significant insight into the functional roles of monocyte subsets in humans.

<div align="center">Murine Monocyte Heterogeneity</div>

Murine monocyte subsets are not distinguishable by CD14 and CD16 antigen expression (Serbina et al., 2008), but instead are recognized as CD115$^+$ (macrophage colony stimulating factor [M-CSF] receptor), CD11b$^+$, and F4/80^{int} blood cells (Ingersoll et al., 2010). Two subsets of monocytes have been described in mice, which can be identified based upon their relative expression of the pro-inflammatory monocyte marker Ly-6C (Sunderkotter et al., 2004). Ly-6C can be identified by flow cytometry using the Gr-1 antibody, which recognizes an epitope of Ly-6C (Fleming, O'hUigin, & Malek, 1993). It is now accepted that mouse monocyte subsets are grouped as Ly-6C$^+$ (further divided as Ly-6C^{high} and Ly-6C^{middle}), and Ly-6C^{lo} or Ly-6C$^-$ cells

<div align="center">16</div>

(Yang, Zhang, Yu, Yang, & Wang, 2014). Ly-6C$^+$ mouse monocytes are characterized by the CX$_3$CR1^{low}CCR2$^+$CD62L$^+$(L-Selectin) phenotype, and are suggested to correspond to classical (CD14^{++} CD16$^-$) human monocytes (Taylor & Gordon, 2003). Ly-6C^{high} and Ly-6C^{middle} subsets express high levels of CCR2 and low levels of CX$_3$CR1 (Saederup et al., 2010). MCP-1, an inflammatory cytokine, which signals through C-C chemokine receptor 2 (CCR2) on Ly-6C$^+$ monocytes, has been suggested to induce a conformational change in the formation of specific surface adhesion receptors on these cells, resulting in high-affinity vascular binding and subsequent transmigration to areas of tissue damage (Yang et al., 2014). This role is consistent with the Ly-6C$^+$ cells pro-inflammatory functions, and potent capacity for phagocytosis (Serbina et al., 2008). During early inflammation, Ly-6C$^+$ cells are thought to enter damaged tissue where they preferentially differentiate into M1 pro-inflammatory macrophages, which secrete pro-inflammatory cytokines and exhibit phagocytic and proteolytic inflammatory functions (Ginhoux & Jung, 2014). Additionally, the short half-life of Ly-6C+ monocytes (approx. 8 hours), likely limits the function of this cell to that of a macrophage precursor (Italiani & Boraschi, 2014).

Ly-6C$^-$ mouse monocytes are characterized by the CX$_3$CR1^{high}CCR2$^-$CD62L$^-$(L-Selectin) phenotype, express low levels of CCR2 and high levels of CX$_3$CR1 (Saederup et al., 2010). A distinction within mouse Ly-6C$^-$ monocytes that corresponds to human non-classical and intermediate distinctions not yet been characterized; however, Ly-6C$^-$ monocytes are suggested to correspond to human CD16$^+$ monocytes (Ingersoll et al., 2010; Taylor & Gordon, 2003). Indeed, a recent study demonstrated the presence of FcγR (receptor corresponding to CD16) on Ly-6C$^-$ monocytes, but not Ly-6C$^+$ monocytes (Santiago-Raber et al., 2009). In comparison to the Ly-6C$^+$ monocytes, Ly-6C$^-$ monocytes have a circulating half-life of approximately 5-7 days (Yona et al., 2013), suggesting that this cell likely has a functional role within the circulation that

is distinct from that of the Ly-6C+ monocyte. Previous studies suggest that Ly6C^{-} monocytes are recruited to tissue during the latter phase of inflammation, where they subsequently develop into resident macrophages (Geissmann et al., 2003), and thus represent the end stage of a distinct differentiation path of a common precursor cell. This is consistent with the longer half-life of this cell, since this Th2/M2 anti-inflammatory response does not peak until 4 days post-injury (Tidball & Villalta, 2010). However, bone marrow derived monocytes in mice appear to be exclusively Ly-6C positive (Sunderkotter et al., 2004). Consistent with this, a recent fate mapping study provides evidence to suggest that Ly6C^{+} blood monocytes constitute an obligatory steady-state precursor to Ly6C^{-} monocytes, with subsequent maturation of the Ly6C^{+} cell resulting in a dichotomous phenotype (Yona et al., 2013). A similar shift in cell phenotype has also been reported in Ly-6C^{+} monocyte derived M1 pro-inflammatory macrophages (Arnold et al., 2007). These cells appear to have the ability to acquire the phenotypic features and functional properties of M2 anti-inflammatory macrophages within inflamed skeletal muscle. Together, these observations lend credence to the hypothesis that distinct monocyte/macrophage subsets represent subsequent maturation stages in a common path of differentiation, which likely leads to divergent functional properties (Stansfield & Ingram, 2015). This hypothesis dictates that, in the absence of inflammation, Ly-6C^{+} monocytes differentiate into Ly-6C^{-} monocytes, which remain in the circulation for up to two weeks (Yona et al., 2013). While in circulation, the primary function of the Ly-6C^{-} cell appears to be patrolling the endothelium and monitoring its integrity (Wermuth & Jiminez, 2015). It has also been suggested that in addition to this patrolling function, Ly-6C^{-} monocytes may also serve to replenish the resident macrophage population (Geissmann et al., 2003). The functional properties of each murine monocyte subset are summarized in Figure 3. Briefly, Ly-6C^{+} monocytes appear to be preferentially and rapidly

recruited to sites of injury and infection in response to pro-inflammatory cues such as MCP-1.

Most inflammatory monocytes are thought to differentiate into macrophages, which are

important for the clearance of tissue debris and for the resolution of inflammation. In contrast, in

the absence of inflammation, Ly-6C⁻ monocytes patrol the endothelial surface and coordinates its

repair via recruitment of neutrophils as required (Ginhoux & Jung, 2014), and are hypothesized

to precede a source of tissue resident macrophages/dendritic cells (Taylor & Gordon, 2003).
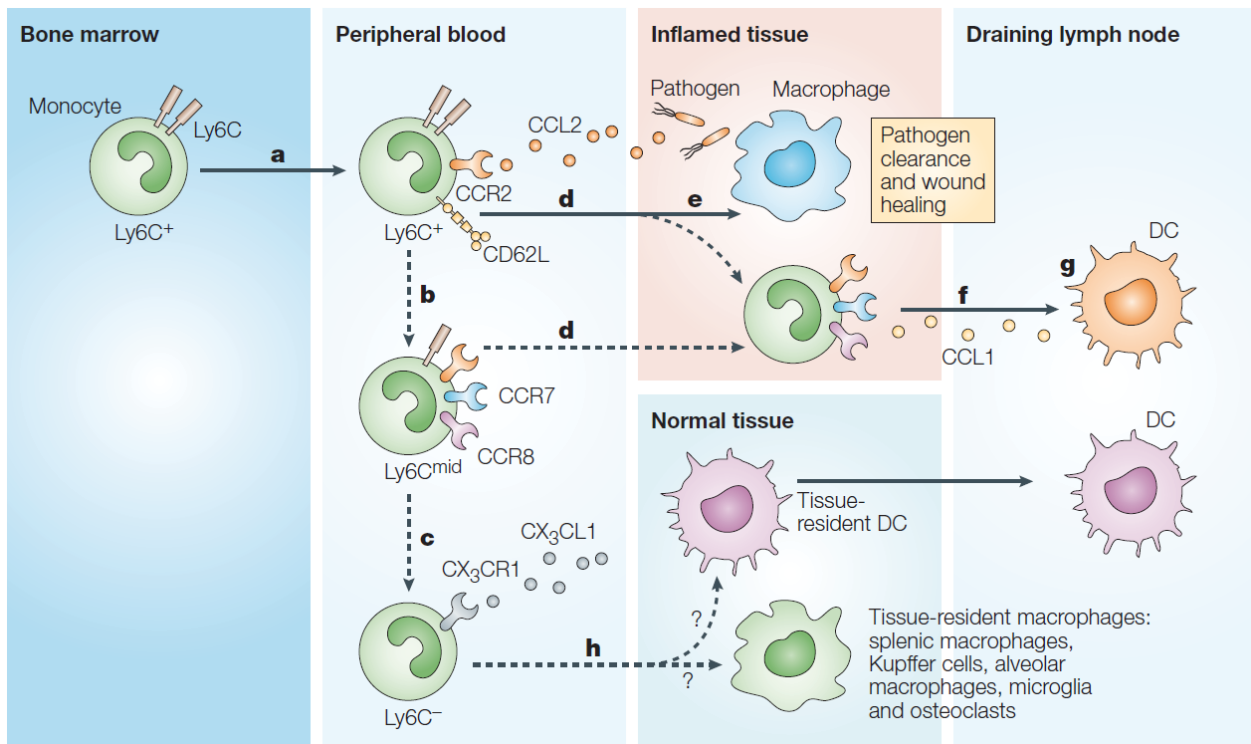
Figure 3. Development and function of monocyte subsets in mice.
Monocyte and Macrophage Heterogeneity. Gordon & Taylor (2005). CX$_3$CL1, CX$_3$C-chemokine ligand 1 (fractalkine); CCR7, CC-chemokine receptor 7; CCR8, CC-chemokine receptor 8. Included with permission from Elsevier; License #: 3582601104753.

Monocyte Heterogeneity in Humans

In humans, classical monocytes express high levels of CD14 antigen and no CD16 antigen (Crowe & Ziegler-Heitbrock, 2010; Weber et al., 2000; Yang et al., 2014). These cells constitute the most prevalent monocyte population, accounting for approximately 80-95% of total monocytes in human blood (Strauss-Ayali et al., 2007; Wong et al., 2011; Yang et al., 2014). Gene enrichment in gene ontology biologic processes, which enables the delineation of unique characteristics that are inherent to monocyte subsets through the analysis of gene expression, suggest that classical monocytes exhibit significant enrichment in the categories of angiogenesis, wound healing, and coagulation (Wong et al., 2011). Additionally, $CD14^{++}CD16^{-}$ cells are reported to respond to bacterial components, toxins, drugs, hypoxia, nutrient levels and hormones, while the pro-inflammatory mediators *S100A12* and *S100A9* and *S100A8* (calcium binding proteins) are among the top 50 most highly expressed genes for this subset (Wong et al., 2011). These data suggest that classical monocytes are highly versatile and capable of responding to a variety of external cues, making them ideal for mediating tissue repair or immune functions. These functions are consistent with the reports of others who suggest that macrophages derived from classical monocytes exhibit excellent phagocytic function (Grage-Griebenow et al., 2000; Grage-Griebenow, Flad, & Ernst, 2001). Further, the early inflammatory response appears to be dominated by the selective recruitment of classical monocytes to injured tissue (Ingersoll, Platt, Potteaux, & Randolph, 2011; Wermuth & Jiminez, 2015), where they infiltrate at sites of damage/inflammation in response to damage associated molecular patterns (DAMP's), and subsequently differentiate into inflammatory M1 macrophages (Soehnlein & Lindbom, 2010; Wermuth & Jiminez, 2015) for the purpose of phagocytizing opsonized cellular debris (Yang et al., 2014). This function is further demonstrated through the expression of

specific surface adhesion (CD11b) and chemokine receptors (CCR2) (Stansfield & Ingram, 2015). This pattern of cell surface apparatus suggests that $CD14^{++}CD16^-$ monocytes are equipped to respond to inflammatory signals, allowing for their subsequent migration and adhesion to the endothelial wall in areas of tissue damage (Imhof & Aurrand-Lions, 2004; Mantovani, 1999; Starikova, Lebedeva, & Freidlin, 2010).

Non-classical monocytes are characterized by their relative low expression of the CD14 antigen, and a high expression of the CD16 antigen. Under the former nomenclature, non-classical monocytes (consisting of non-classical and intermediate monocytes) accounted for approximately 2-11% of the total monocyte population in human blood (Strauss-Ayali et al., 2007; Yang et al., 2014). More recent research by Wong and colleagues (2011) suggest that non-classical monocytes account for 9.2% ± 4.4% of total circulating monocytes. These cells are considered the major pro-inflammatory monocyte subpopulation and are the major producers of tumor necrosis factor in human blood (Belge et al., 2002; Cros et al., 2010; Passlick, Flieger, & Ziegler-Heitbrock, 1989; Wong et al., 2011). Similar to $Ly\text{-}6C^-$ murine monocytes, non-classical human monocytes have been observed to demonstrate a patrolling behavior in healthy tissues through long-range crawling on the resting endothelium, and thus may represent a marginal pool of monocytes. This may allow for rapid tissue invasion in the event of damage or infection (Auffray et al., 2007; Cros et al., 2010). However, following strenuous anaerobic exercise, these non-classical monocytes have been observed to mobilize from the marginal pool and enter into the circulation, contributing to a 4.8 fold increase in circulating cells within an order of minutes (Steppich et al., 2000). This compartment shift may be the result of shear stress or an interaction with catecholamines (Freidenreich & Volek, 2012), but nevertheless precludes the rapid entry of these cells into sites of exercise-induced damage. Consistent with this, the human $CD14^+CD16^{++}$

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non-classical subpopulation has been shown to exhibit poor phagocytic capacity (Cros et al., 2010), and reduced ROS production, which is a key aspect of phagocytic function in the destruction of cellular debris (Cros et al., 2010; Szaflarska et al., 2004). This subset of monocytes also exhibits higher expression of CX_3CR1, a chemokine receptor reported to mediate resident macrophage accumulation in non-inflamed tissue (Ancuta et al., 2003; Geissmann et al., 2003; Landsman et al., 2009), and exhibit significantly lower levels of CCR2, the chemokine receptor mediating monocyte chemotaxis during inflammation (Wong et al., 2011). Consequently, non-classical monocytes fail to migrate towards the CCR2 ligand MCP-1 (Weber et al., 2000). These cells have also been reported to strongly resemble mature tissue resident macrophages (H. W. Ziegler-Heitbrock et al., 1993), which is consistent with a two-fold greater affinity for the endothelium in the absence of inflammation, compared to the classical monocyte (Starikova et al., 2010). Moreover, non-classical monocytes have recently been shown to exhibit a significantly lower expression of CD11b receptor compared to classical monocytes (Wong et al., 2011), which has been shown to be critical for tissue repair responses following skeletal muscle damage (Arnold et al., 2007).

The exact role of non-classical monocytes remains unclear; however, these cells do not appear to be involved in the acute response to tissue inflammation. While recent reviews have focused upon characterizing the origin of tissue resident macrophages (Ginhoux & Jung, 2014; Italiani & Boraschi, 2014); to date, no study has provided conclusive evidence against a possible role for the non-classical monocyte in tissue macrophage homeostasis (Italiani & Boraschi, 2014). The origin of tissue resident macrophages appears to vary considerably between tissues, and while the origin of these cells has been somewhat characterized in CNS, dermis, epidermis, lung, heart, kidney, pancreas, liver, spleen and peritoneal tissue (Epelman et al., 2014; Ginhoux

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et al., 2010; Guilliams et al., 2013; Schulz et al., 2012), the origin of skeletal muscle resident macrophages has not yet been determined.

Monocyte Chemotaxis

Skeletal muscle repair is a tightly regulated process consisting of an initial inflammatory response, followed by subsequent regeneration and revascularization of damaged tissue (H. Lu et al., 2011; Tidball, 2005). The initial inflammatory response is characterized by a rapid and sequential invasion of leukocyte populations for the purpose of reconstituting tissue integrity. Members of the chemokine family of chemotactic cytokines play a fundamental functional role in this process by directly influencing the vascular migration and accumulation of specific leukocytes to sites of tissue injury (Furie & Randolph, 1995; Henningsen, Pedersen, & Kratchmarova, 2011). Within healthy skeletal muscle, the chemokine expression profile is fairly limited (De Paepe & De Bleecker, 2013); however, in response to tissue damage, the secretion of specific chemokines is induced or significantly upregulated within the tissue (Hirata et al., 2003). Chemokine effects are mediated via binding with heptahelical receptors located on the plasma membrane of leukocytes, which are coupled to GTP binding proteins (Baggiolini & Loetscher, 2000). Consequently, the trafficking behavior of specific leukocyte subsets during inflammation is dependent upon the both the presence of specific chemokines, and the adequate expression of its respective receptor.

Several studies suggest that a number of chemokines, including MCP-1 and MCP-3 mediate the chemotaxis of classical monocytes to damaged tissue (Charo & Ransohoff, 2006; Rossi & Zlotnik, 2000), raising the question as to which ligand the monocyte is in fact

responding to. Observational studies report an increase in MCP-1 that parallels the early

inflammatory response following induced muscle ischemia (Shireman, Contreras-Shannon,

Reyes-Reyna, Robinson, & McManus, 2006). In accordance, loss of MCP-1 alone via targeted

disruption of *SCYA2* (the gene encoding MCP-1) has been shown to sufficiently impair

monocyte trafficking in a number of different inflammatory models (B. Lu et al., 1998). These

findings were corroborated in later work by Shireman and colleagues (2007), where an altered

inflammatory response, delayed restoration of perfusion, and significant decrements in indices of

muscle regeneration were observed following femoral artery excision induced muscle damage in

MCP-1 knockout mice. Nevertheless, selective deletion of MCPs using homologous

recombination in embryonic stem cells reveal significant deficits in monocyte recruitment in

both MCP-1 and MCP-3 knockout models (Tsou et al., 2007). This is consistent with the

observations of others (Jia et al., 2008), highlighting a possible role for MCP-3 in monocyte

chemotaxis, and a potential synergy between MCP-1 and MCP-3 for the optimal recruitment of

classical monocytes to damaged tissue.

Notwithstanding, recent evidence suggests that MCP-1 is in fact the primary chemokine

required for classical monocyte recruitment, and not MCP-3 (M. Takahashi, Galligan,

Tessarollo, & Yoshimura, 2009). Takahashi and colleagues (2009) report a marked increase in

MCP-3 mRNA production following deletion of a 2.3-kb genomic DNA sequence from the

MCP-1 gene (that resulted in MCP-1 deficiency), which was not apparent in previously

generated MCP-1 knockout mice (MCP-1$^{-/-}$) or MCP-1$^{+/+}$ controls. Comparison of MCP-1

deficient and MCP-1$^{-/-}$ mice revealed no significant differences in monocyte recruitment,

indicating that MCP-3 was unable to compensate for the loss of MCP-1 (M. Takahashi et al.,

2009). It is likely that MCP-3 plays a limited role in monocyte recruitment. Consequently, MCP-1 signaling appears to be the primary ligand mediating the recruitment of classical monocytes.

CCR2 signaling appears to have an important role in the recruitment of monocytes to injured tissue (B. Lu et al., 1998; Shireman et al., 2006; Shireman et al., 2007). CCR2 is considered to be the exclusive receptor of MCP-1 (Charo & Ransohoff, 2006; Gerard & Rollins, 2001), and while reports suggest that neutrophil and lymphocyte recruitment is similar between wild type and CCR2 knockout mice ($CCR2^{-/-}$) (H. Lu et al., 2011), the mobilization of monocytes both from the bone marrow to the blood, and from the blood to the injured tissue, is significantly impaired in $CCR2^{-/-}$ mice compared to controls (H. Lu et al., 2011). This impairment appears to be directly associated with classical monocytes. Analysis of gene expression reveals that CCR2 is almost exclusively expressed by the classical $CD14^{++}CD16^{-}$ monocyte population in humans (Wong et al., 2011). Further, selective labeling of monocyte subsets indicate that only $Ly\text{-}6C^{+}$ monocytes are recruited to damaged tissue (Arnold et al., 2007). While the appearance of $Ly\text{-}6C^{-}$ monocytes in the blood is reported to be similar between $CCR2^{-/-}$ and control mice (H. Lu et al., 2011), both $Ly\text{-}6C^{+}$ and $Ly\text{-}6C^{-}$ monocyte/macrophage subsets appear to be drastically reduced within the injured muscle of $CCR2^{-/-}$ mice. This provides additional evidence favoring the differentiation of $Ly\text{-}6C^{+}$ monocyte derived pro-inflammatory macrophages into anti-inflammatory macrophages, in lieu of a secondary recruitment of $Ly\text{-}6C^{-}$ monocytes in the later stages of tissue repair (Arnold et al., 2007; H. Lu et al., 2011). Nevertheless, $CCR2^{-/-}$ mice exhibit a markedly reduced inflammatory response following tissue injury, resulting in significantly impaired muscle regeneration, fibrosis, fat infiltration, and calcification of the tissue (H. Lu et al., 2011; Warren et al., 2005). These observations are consistent with other CCR2 knockout studies, which report marked reductions in monocyte

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infiltration, along with concomitant deficits in both muscle regeneration and tissue revascularization following injury (Arnold et al., 2007; Brodmerkel et al., 2005; Chazaud et al., 2003; Contreras-Shannon et al., 2007; Ochoa et al., 2007; Tsou et al., 2007; Volpe et al., 2012; Warren et al., 2004; Willenborg et al., 2012). Interestingly, the role of macrophage produced IGF-1 in MPC growth discussed earlier, appears to be monocyte derived and dependent upon CCR2 signaling (H. Lu et al., 2011). Together, these data suggest that MCP-1/CCR2 signaling is critical for classical monocyte egress from the bone marrow (Serbina & Pamer, 2006), and for subsequent recruitment from the blood to the tissue, and for optimal tissue regeneration.

Recent research suggests a role for resident macrophages in orchestrating this immune cell response to myofiber injury (Brigitte et al., 2010). Located within the epimysium and perimysium of the muscle, resident macrophages appear to contribute to an extensive recruitment of neutrophils and monocytes from the circulation through selective secretion of neutrophil and monocyte chemotactic proteins. The functional role of resident macrophages in monocyte recruitment is consistent with the dramatic reduction in monocyte infiltration observed following selective depletion of resident macrophages in injured muscle (Chazaud et al., 2009). The processes of monocyte recruitment into skeletal muscle following injury is depicted in figure 4. Briefly; following muscle damage, activated myogenic cells secrete a number of chemotactic factors, including MCP-1, aimed at monocyte recruitment. However; compared to that of myogenic cells, resident macrophage recruitment of monocytes constitutes a substantially more robust chemotactic pathway, initiating the recruitment of both neighboring resident macrophages and circulating monocytes in an MCP-1 dependent pathway.
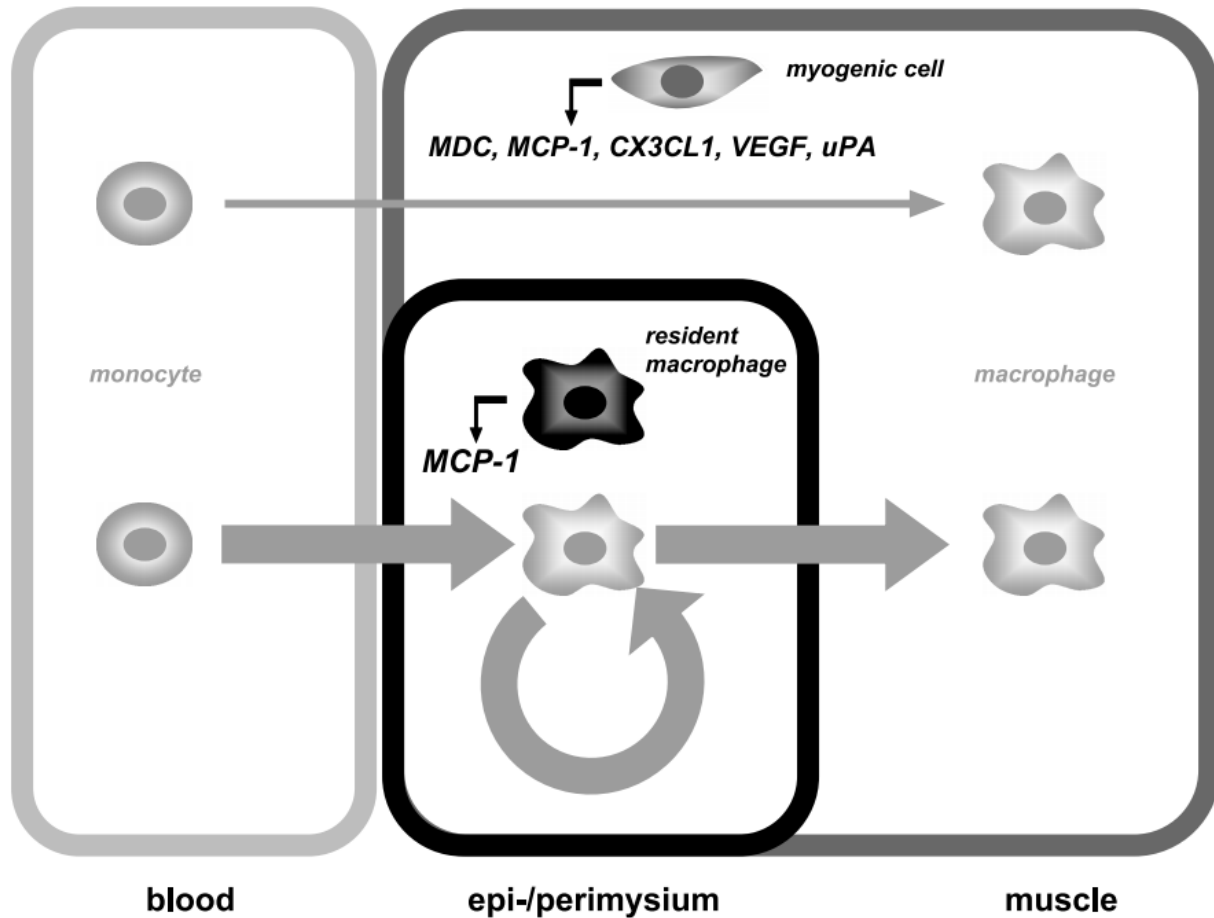
Figure 4. Monocyte recruitment into skeletal muscle following injury.
Dual and beneficial roles of macrophages during skeletal muscle regeneration. Chazaud et al.
(2009). MDC, macrophage–derived chemokine; MCP-1, monocyte chemoattractant protein 1;
CX_3CL1, fractalkine; VEGF, vascular endothelial growth factor; uPA, urokinase-type
plasminogen activator. Included with permission from Wolters Kluwer Health; License #:
3590431459002.

Recent reports suggest that CCR2 does not appear to demonstrate a desensitization to the

MCP-1 ligand following prolonged exposure (Volpe et al., 2012). However, it has previously

been posited that the expression of chemotactic receptors on leukocytes can be modulated by

external environmental cues in order to fine tune the timing and magnitude of the inflammatory

response (Burnett, 1992; Fantuzzi et al., 1999). This suggests that the presence of certain ligands

within the circulation may precipitate an up- or down-regulation of chemotactic receptors. Changes in the expression of the CCR2 receptor and/or the secretion of the MCP-1 ligand may therefore have a profound impact on monocyte recruitment, which may ultimately influence muscle regenerative processes.

Glucocorticoid/Monocyte Interactions

Resistance exercise is a potent stimulus for acute increases in the concentrations of circulating hormones (Smilios et al., 2003). In particular, significant elevations in circulating cortisol are observed following resistance exercise of sufficient volume and intensity (Ahtiainen et al., 2003). Cortisol accounts for 95% of secreted glucocorticoids in humans, which are released from the adrenal cortex in response to the stress of exercise (Kraemer & Ratamess, 2005). The constitutive expression of glucocorticoid receptors (GCR) on human peripheral blood mononuclear cells (PBMCs) (Bartholome et al., 2004), as well as the potential for these receptors to up-regulate in response to immunostimulation, has previously been documented (Bartholome et al., 2004; Spies et al., 2007). Consequently, the expression of GCR on monocytes provide the molecular basis for these cells to be targets of cortisol signaling prior to, during, and following resistance exercise.

Resent research suggests that cortisol's regulation of innate immunity may be both pro- and anti-inflammatory (Sorrells & Sapolsky, 2010; Yeager, Pioli, & Guyre, 2011). Consistent with this, glucocorticoids have been shown to modulate the expression of the pro-inflammatory receptor CCR2 on human monocytes *in vitro* (Okutsu et al., 2008; Penton-Rol et al., 1999; Pettersson et al., 2005). Penton-Rol et al. (1999) report a selective up-regulation of CCR2 mRNA

expression following treatment of human monocytes with the corticosteroid dexamethasone (Penton-Rol et al., 1999). In lieu of an increased rate of nuclear transcription, the corticosteroid treatment was observed to augment the CCR2 mRNA half-life. This effect was associated with increased chemotaxis towards MCP-1, as measured using a chemotaxis micro-chamber *in vitro*, and was abolished in the presence of glucocorticoid receptor agonist RU486. This indicates that the up-regulation of CCR2 was mediated by GCR.

Similar findings have been observed by Okutsu and colleagues (2008). Human PBMC's from untrained men incubated with either serum drawn immediately and 2 hours post-exercise (60 minutes cycling at 70% VO_{2peak}) or cortisol alone, exhibit a dose- and time-dependent increase in CCR2 expression *in vitro*. This expression was 2.4 and 1.3 times higher than that of the control condition, respectively. Additionally, the up-regulation of CCR2 was achievable with the lower limit of physiological plasma cortisol concentration. Similar to the findings of Penton-Rol and colleagues (1999), the effects of cortisol and serum on CCR2 were abolished in the presence of the GCR agonist RU486. Notwithstanding, the exercise stimulus was not sufficient to cause an up-regulation of CCR2 *in vivo.*

Taken together, examinations of the effects of glucocorticoids on monocyte CCR2 expression *in vitro* suggest that cortisol, and other corticosteroids may augment the chemotactic capacity of these cells to inflammatory stimuli. Notwithstanding, the up-regulation of CCR2 in the presence of glucocorticoids *in vitro* requires at least 4-6 hours incubation time (Okutsu et al., 2008; Penton-Rol et al., 1999). Consequently, *in vitro* observations may not translate to the up-regulation of CCR2 *in vivo.* This is consistent with the absence of *in vivo* changes to CCR2 expression following cycling activity (Okutsu et al., 2008). The effects of resistance exercise on the expression of CCR2 *in vivo* is yet to be determined.

Monocyte Adhesion

In order for monocytes to successfully migrate into damaged tissue, they must first make contact with the endothelium immediately adjacent to it. However, the shear stress associated with blood flow *in vivo* make this contact functionally complex. Therefore, monocyte transendothelial migration requires a series of sequential steps. These steps include capture, rolling, activation, arrest, adhesion strengthening, and intravascular (intraluminal) crawling, that together, ultimately lead to paracellular or transcellular monocyte transmigration (Ley, Laudanna, Cybulsky, & Nourshargh, 2007; Schenkel et al., 2004). Each step in the process is a fundamental pre-requisite for the next step, in what is known as the leukocyte adhesion cascade. The leukocyte adhesion cascade has previously been described in detail by Ley and colleagues (2007) and is depicted in Figure 5. Several leukocyte specific structural families of adhesion molecules are known to mediate each step within the cascade. However; for the purposes of this review, only molecules known to directly mediate intravascular crawling will be discussed. This step is the direct pre-requisite to monocyte transmigration.

Prior to crossing the walls of the post-capillary venules, monocytes are observed to crawl inside the blood vessels (intravascular crawling), seeking preferred sites of transmigration (Ley et al., 2007). Intravascular crawling is predominantly mediated by cytoskeletally regulated heterodimers called integrins (Schenkel et al., 2004; Sumagin, Prizant, Lomakina, Waugh, & Sarelius, 2010), which interact with their constitutive or inducible endothelial ligands (Laudanna & Alon, 2006). Under homeostatic conditions, integrins are maintained in a low-affinity conformational state (Shimaoka et al., 2003). However; in the presence of inflammation, select chemokines are secreted, which serve to activate/enhance the affinity and/or surface expression

of integrins (Ley et al., 2007). This subsequently increases the ligand binding capacity of the cell (Constantin et al., 2000; Montresor, Toffali, Constantin, & Laudanna, 2012; Sun et al., 2014), while also facilitating a marked decrease in the rate of ligand dissociation (Ley et al., 2007). Cumulatively, these effects lead to the augmentation of endothelial adhesion.

Integrins exist as two non-covalently bound alpha and beta subunits, which pair to form a heterodimer. Each heterodimer consists of a large extracellular domain capable of binding proteins in the extracellular environment (Hynes, 2002). The functional specificity of these molecules resides largely within the extracellular domain of the molecule (Xiong, Chen, & Zhang, 2003; Zen et al., 2011). CD11a and CD11b are alpha glycoprotein chains, which combine with the beta chain CD18 to form the extracellular domain of β_2 integrin receptors LFA-1 (Lymphocyte function-associated antigen 1; CD11a/CD18) and CR3 (cell adhesion molecule complement receptor 3; Mac-1; CD11b/CD18) (Ehlers, 2000). Both CD11a and CD11b are reported to have functional roles in monocyte crawling; however, CD11b mediated crawling is reported to predominate during inflammation (Sumagin et al., 2010).

CD11b is expressed exclusively on granulocytes and monocytes (Dunne, Ballantyne, Beaudet, & Ley, 2002). The counter-ligand/receptor for CD11b is recognized as intracellular adhesion molecule-1 (ICAM-1) (Diamond et al., 1990), and is upregulated on the surface of inflamed endothelial cells following tissue injury (Paulis et al., 2012). Pursuant to damage, the intravascular crawling behavior of monocytes is reported to occur in a CD11b/ICAM-1 dependent manner (Dunne, Collins, Beaudet, Ballantyne, & Ley, 2003; Schenkel et al., 2004). Ligation of ICAM-1, especially under conditions of high ICAM-1 expression, is thought to trigger cytoplasmic signaling events that lead to the translocation of apical ICAM-1, allowing the extension of leukocyte membrane protrusions into endothelial-cell junctions (Schenkel et al.,

2004). This response is thought to result in the formation of channels through which the

monocyte can migrate. Consistent with this role, the delivery of a monoclonal antibody directed

against an epitope of the CD11b molecule *in vivo*, has been shown to inhibit monocyte

recruitment to inflammatory stimuli (H. Rosen & Gordon, 1987). Additionally, it has recently

been shown that suppression of the CD11b molecule *in vitro* results in a concomitant decrease in

endothelial adhesion (Arakawa et al., 2010; Mesri et al., 1998). Further, blockade of CD11b in

conjunction with LFA-1 has been shown to result in impaired cell movement, and the inability of

monocytes to reach endothelial cell junctions (Schenkel et al., 2004). Consequently, it appears

that CD11b is vital for the adhesion of monocytes in response to environmental inflammatory

cues.
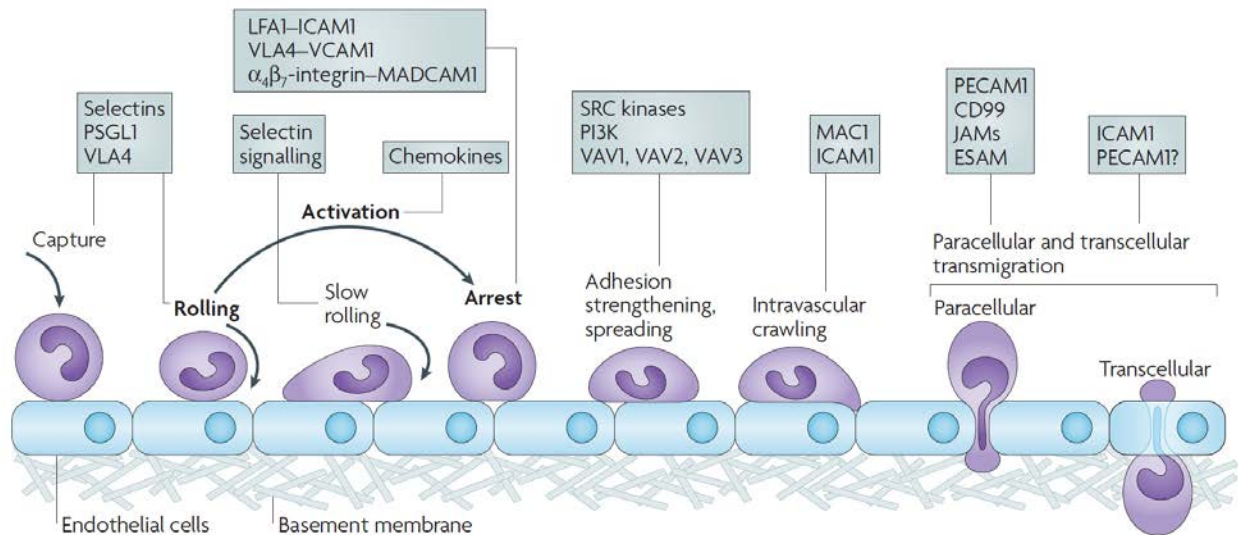
Figure 5. The leukocyte adhesion cascade.
Getting to the site of inflammation: the leukocyte adhesion cascade. Ley et al. (2007). ESAM, endothelial cell-selective adhesion molecule; ICAM1, intercellular adhesion molecule 1; JAM, junctional adhesion molecule; LFA1, lymphocyte function-associated antigen 1 (also known as αLβ2-integrin); MAC1, macrophage antigen 1; MADCAM1, mucosal vascular addressin cell-adhesion molecule 1; PSGL1, P‑selectin glycoprotein ligand 1; PECAM1, platelet/endothelial-cell adhesion molecule 1; PI3K, phosphoinositide 3‑kinase; VCAM1, vascular cell-adhesion molecule 1; VLA4, very late antigen 4 (also known as α4β1-integrin). Included with permission from Nature Publishing Group; License #: 3592000371954.

In accordance with these reports, an up-regulation in the expression of the CD11b has previously been linked with the increased ability of monocytes to adhere to endothelial cells (Weber, Erl, & Weber, 1995). Increases in CD11b expression on monocytes has previously been reported following long distance running (Nielsen & Lyberg, 2004), and high-volume resistance training (Gonzalez et al., 2014; Jajtner et al., 2014). This up-regulation has previously been associated with increased circulating MCP-1 (K. Takahashi et al., 2003; Vaddi & Newton, 1994) and TNFα (Gamble et al., 1985; Montecucco et al., 2008). MCP-1 and/or TNFα mediated up-regulation of CD11b expression may augment the ability of monocytes to adhere to the endothelium in areas of damage, which could ultimately lead to a higher rate of infiltration, and subsequent modulation of tissue adaptation.

Conclusions

Together, murine and human *in vivo*, as well as *in vitro* examinations of monocyte/macrophage responses to tissue damage/inflammation, have shed considerable light on the integrative function of distinct immune cells in the resolution of tissue inflammation. Following tissue injury, there is a predictable series of responses by innate immune system. While the duration and intensity of certain events may change, the systematic activation and redistribution of leukocyte subsets in response to injury is fairly consistent. Pursuant to a disturbance in tissue homeostasis, patrolling non-classical monocytes and resident macrophages are among the first cells to respond. These cells are able to sense the disturbance in homeostasis, and respond by rapidly producing cytokines and chemokines, which subsequently leads the activation and expression of specific receptors on endothelial cells and leukocytes. These

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receptors may also be responsive to endocrine signals. Neutrophils are the first cell to invade the

site of injury, where they release granule contents that modify the damaged tissue and promote

the extravasation of classical inflammatory monocytes. The life-span of emigrated neutrophils is

rather short and is subject to modification by pro- or anti-apoptotic signals, some of which are

produced by macrophages. Macrophages and apoptotic neutrophil signals prevent further

infiltration of neutrophils, but continue to signal classical monocytes and promote their influx.

Classical monocytes subsequently enter the tissue and differentiate into M1 pro-inflammatory

macrophages, which function to phagocytize opsonized cellular debris and apoptotic neutrophils

while stimulating MPC proliferation. The eventual clearance of apoptotic neutrophils and

cellular debris promotes a shift in macrophage phenotype from a pro-inflammatory M1 to an

anti-inflammatory M2 phenotype, which leads to the release of specific growth factors that

ultimately modulate to the reconstitution of tissue homeostasis. The sequential activation and

function of immune cells to inflammatory cells are summarized in figure 6.
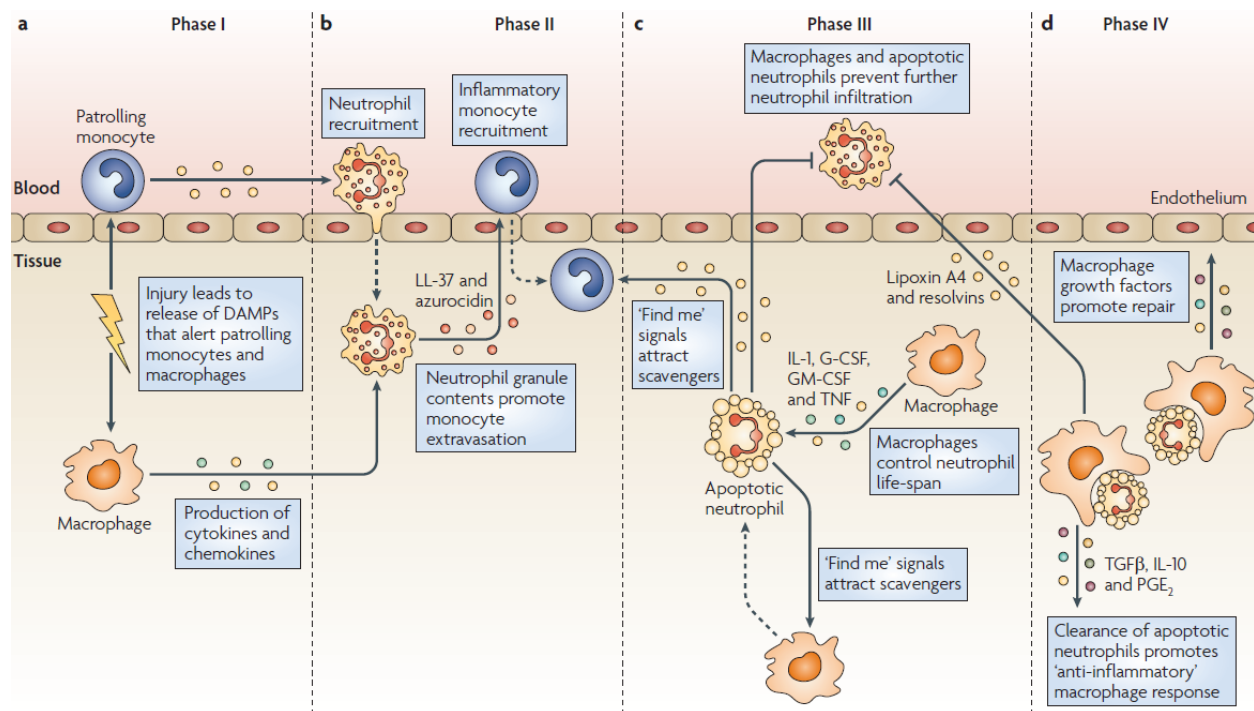
Figure 6. Phagocyte interactions in inflammation.
Phagocyte Partnership during the Onset and Resolution of Inflammation. Soehnlein & Lindbom (2010). IG-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte–monocyte colony-stimulating factor; IL, interleukin; PGE2, prostaglandin E2; TGFβ, transforming growth factor-β;TNF, tumor necrosis factor. Included with permission from Nature Publishing Group; License #: 3582600880056.

It is currently unclear whether the acute inflammatory response differs between various resistance exercise protocols. However, given that cortisol has been shown to have the ability to modulate monocyte receptor expression *in vitro*, and the systemic profile of cortisol is known to be significantly different between high-intensity and high-volume resistance exercise protocols, it is feasible to suggest that systemic immune response may differ between these exercise protocols. An increase in monocyte chemotaxis and adhesion increase the likelihood of monocyte extravasation, which may subsequently contribute to an augmented macrophage pool within the muscle. Since macrophages directly support both the clearance of cellular debris, and

the growth of new muscle, training paradigms that maximize the potential for monocyte

extravasation may ultimately lead to greater tissue adaptations.

CHAPTER III: METHODOLOGY

Participants

Ten resistance-trained men (90.1 ± 11.3 kg; 176.0 ± 4.9 cm; 24.7 ± 3.4 yrs; 14.1 ± 6.1% body fat) were recruited to participate in this randomized, cross-over design research study. Strict recruitment criteria were implemented to increase homogeneity of the sample. Inclusion criteria required participants to be between the ages of 18 and 35 years, with a minimum of one year of resistance training experience, and the ability to squat a weight equivalent to their body mass. Participants had 6.7 ± 4.6 years of resistance training experience with an average maximum barbell back squat of 172.7 ± 25.2 kg. All participants were free of any physical limitations that may have affected performance. Additionally, all participants were free of any medications and performance enhancing drugs, as determined by a health and activity questionnaire. Following an explanation of all procedures, risks, and benefits, each participant provided his informed consent prior to participation in this study. The research protocol was approved by the New England Institutional Review Board prior to participant enrollment.

Maximal Strength Testing

Prior to experimental trials, participants reported to the Human Performance Laboratory (HPL) to establish maximal strength (1RM) on all lifts involved in the exercise protocol. Participants performed a standardized warm-up consisting of five minutes on a cycle ergometer against a light resistance, 10 body weight squats, 10 body weight walking lunges, 10 dynamic walking hamstring stretches, and 10 dynamic walking quadriceps stretches. Following the

warm-up, 1RM testing for the barbell back squat and leg press were performed using methods

previously described (Hoffman, 2006). Briefly, each participant performed two warm-up sets

using a resistance of approximately 40-60% and 60-80% of his perceived maximum,

respectively. For each exercise, 3-4 subsequent trials were performed to determine the 1RM. A

3-5 minute rest period was provided between each trial. Maximum strength testing was

administered by the same Certified Strength and Conditioning Specialist to ensure that each

participant reached the parallel position for each repetition of the squat and that the exercise

technique was consistent between sessions. For all other exercises, the 1RM was assessed using a

prediction formula based on the number of repetitions performed to fatigue using a given weight

(Brzycki, 1993). Trials not meeting the range of motion criteria for each exercise or where

proper technique was not used were discarded.

Experimental Trials

On the morning of each trial, participants reported to the HPL after a 10-hour overnight

fast and having refrained from all forms of moderate to vigorous exercise for the previous 72

hours. Experimental trials were performed in a balanced, randomized order, and each

experimental trial was separated by a minimum of one week to ensure adequate recovery. Each

participant performed experimental trials at the same time of day to avoid the influence of

diurnal variations. During each experimental trial, participants performed the standardized warm-

up routine as described above, followed by a lower-body resistance exercise protocol. Table 1

depicts the volume (VOL) and heavy (HVY) resistance exercise protocols. The VOL protocol

required participants to perform 10 – 12 repetitions with a load of equating to 70% of their 1RM,

with a 1-minute rest period between each set and exercise. The HVY protocol required participants to perform 3 – 5 repetitions with a load equating to 90% of their 1RM with a 3-minute rest period between each set and exercise. Both protocols included six sets of barbell back squats and four sets of bilateral leg press, bilateral hamstring curls, bilateral leg extensions, and seated calf raises. During each protocol, participants were verbally encouraged to complete all repetitions for each set. If the participant was unable to complete the desired number of repetitions, spotters provided assistance until the participant completed the remaining repetitions. Subsequently, the load for the next set was adjusted so that participants were able to perform the specific number of repetitions for each set.

Table 1. Resistance Exercise Protocols

Exercise Order	Volume Protocol (VOL)			Heavy Protocol (HVY)		
	Sets x Repetitions	Intensity	Rest Interval	Sets x Repetitions	Intensity	Rest Interval
1. Barbell Back Squats	6 x 10-12			6 x 3-5		
2. Bilateral Leg Press	4 x 10-12			4 x 3-5		
3. Bilateral Hamstring Curls	4 x 10-12	70% 1-RM	1 Minute	4 x 3-5	90% 1-RM	3 Minutes
4. Bilateral Leg Extensions	4 x 10-12			4 x 3-5		
5. Seated Calf Raise	4 x 10-12			4 x 3-5		

Following each resistance exercise protocol, participants remained in the laboratory for all post-exercise assessments. Blood samples were obtained at six time points over the course of the study: baseline (BL), immediately post-exercise (IP), 30 minutes post-exercise (30P), 1 hour post-exercise (1H), 2 hours post-exercise (2H), and 5 hours post-exercise (5H)

To control for diet, participants were provided a standardized low protein, low carbohydrate breakfast (7 grams protein; 3 grams carbohydrate; 13 grams fat) following BL assessments. Immediately following IP blood sampling, participants were also provided a flavored drink (355 mL, 0 grams protein, 2.5 grams carbohydrates, 0 gram fat). Participants

were permitted to drink water ad libitum during experimental trials, and water consumption was monitored.

Dietary Logs

Participants were instructed to maintain their normal dietary intake leading up to experiment trials. Participants were then instructed to record as accurately as possible everything they consumed during the 24 hours prior to the first experimental trial. For the following experimental trial, participants were required to duplicate the content, quantity, and timing of their daily diet during the 24 hours prior. Participants were instructed not to eat or drink (except water) within 10 hours of reporting to the HPL for experimental trials.

Blood Measurements

During each experimental trial, blood samples were obtained using a Teflon cannula placed in a superficial forearm vein using a three-way stopcock with a male luer lock adapter and plastic syringe. The cannula was maintained patent using an isotonic saline solution (Becton Dickinson, Franklin Lakes, NJ, USA). BL blood samples were obtained following a 15-minute equilibration period. IP blood samples were taken within one minute of exercise cessation. Participants were instructed to lie in a supine position for 15 minutes prior to 30P, 1H, 2H, and 5H blood draws.

All blood samples were collected into three 6 ml Vacutainer® tubes. Blood samples were drawn into either plain, sodium heparin, or K_2EDTA treated tubes. A small aliquot of whole blood was removed and used for determination of hematocrit and hemoglobin

concentrations. The blood in the plain tube was allowed to clot at room temperature for 30 minutes and subsequently centrifuged at 3,000g for 15 minutes along with the remaining whole blood from the other tubes. The resulting serum and plasma was placed into separate micro-centrifuge tubes and frozen at −80°C for later analysis.

Biochemical Analysis

Blood lactate concentrations were analyzed from plasma using an automated analyzer (Analox GM7 enzymatic metabolite analyzer, Analox instruments USA, Lunenburg, MA, USA). Hematocrit concentrations were analyzed from whole blood via microcentrifugation (CritSpin, Westwood, MA, USA) and microcapillary technique. Hemoglobin concentrations were analyzed from whole blood using an automated analyzer (HemoCue, Cypress, CA, USA). Plasma volume shifts were calculated using the formula established by Dill & Costill (1974). To eliminate inter-assay variance, all samples were analyzed in duplicate by a single technician. Coefficient of variation for each assay was 1.4% for blood lactate; 0.4% for hematocrit; and 0.6% for hemoglobin.

Circulating concentrations of testosterone and cortisol were assessed via enzyme-linked immunosorbent assays (ELISA) and a spectrophotometer (BioTek Eon, Winooski, VT, USA) using commercially available kits. Myoglobin concentrations were determined via ELISA (Calbiotech, Spring Valley, CA, USA) and a spectrophotometer. Lactate dehydrogenase (LDH) concentrations were determined via kinetic ELISA (Sigma-Aldrich, St. Louis, MO, USA) and a spectrophotometer. To eliminate inter-assay variance, all samples for each assay were thawed once and analyzed in duplicate in the same assay run by a single technician. Coefficients of

variation for each assay were 4.8% for testosterone; 5.3% for cortisol; 4.1% for myoglobin; and 4.8% for LDH.

Plasma samples were assayed for concentrations of monocyte chemoattractant protein 1 (MCP-1) and tumor necrosis factor-alpha (TNF-α), using a multiplex cytokine assay (Milliplex, Cat no. HCYTOMAG-60K; Millipore, Billerica, MA) on a MAGPIX instrument (Luminex, Austin, TX), according to the manufacturer's instructions. All samples were run in duplicate with a mean intra-assay variance of 8.77% for MCP-1 and 7.00% for TNF-α.

Cell Staining

Cell staining was performed as described previously (Gonzalez et al., 2014; Jajtner et al., 2014; Townsend et al., 2013). Analysis of target receptor expression on CD14^{++}CD16^{-} cells was completed at BL, IP, 1H, 2H and 5H time points. K$_2$EDTA-treated peripheral whole blood was used to identify monocytes, and quantify target receptor expression by direct immunofluorescence and flow cytometry (BD Biosciences, San Jose, CA). Erythrocytes were first lysed from 350 µl of K$_2$EDTA-treated whole blood with BD Pharm Lyse solution (BD Biosciences, San Jose, CA) within 30 min of collection. Samples were then washed in staining buffer containing 1 x phosphate-buffered saline (PBS) and fetal bovine serum (FBS) (BD Pharmingen Stain Buffer; BD Biosciences) followed by centrifugation and aspiration for a total of three washes. Leukocytes were then resuspended in 100 µl BD Pharmingen stain buffer (BD Biosciences). Direct staining methods were used to label CD14 and CD16 (monocyte identifiers), CCR2 (monocyte chemotaxis), CD120a (receptor for TNFα), CD11b (monocyte

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adhesion), and glucocorticoid receptor (GCR). Due to the number of receptors of interest, two separate cell preparations were performed.

For preparation 1, Alexa Fluor® 488 conjugated anti-CD120a (FAB225G, IgG; R&D Systems), allophycocyanin (APC) conjugated anti-CD11b (550019; IgG1; BD Pharmingen™), PerCP Cy5.5 conjugated anti-CD14 (562692; IgG$_{2b}$; BD Pharmingen™) and PE conjugated anti-CD16 (561313; IgG1; BD Pharmingen™) were used in the receptor labeling process. Surface staining for preparation 1 was performed by adding 5µL of directly conjugated Alexa Fluor® 488-anti-CD120a, 20µL of directly conjugated APC-anti-CD11b, 5 µL of directly conjugated PerCP Cy5.5-anti-CD14, and 5 µL of directly conjugated PE-anti-CD16 to the cell suspension, followed by incubation in the dark for 30 minutes at room temperature. Cells were then resuspended in 1.0mL of stain buffer for immediate flow cytometry analysis.

For preparation 2, PerCP Cy5.5 conjugated anti-CD14 (562692; IgG$_{2b}$; BD Pharminigen), allophycocyanin (APC) conjugated anti-CCR2 (FAB151A; IgG$_{2b}$; R&D Systems), and DyLight® 488 conjugated anti-GR (ab139892; IgG; Abcam), were used in the receptor labeling process. Surface staining for preparation 2 was performed by adding 5 µL of directly conjugated PerCP Cy5.5-anti-CD14, and 10 µL of directly conjugated APC-anti-CCR2 to the cell suspension followed by incubation in the dark for 30 minutes at room temperature. Cells were then washed in staining buffer followed by centrifugation and aspiration. Cells were subsequently resuspended in 250 µL of fixation and permeabilization solution (BD cytofix/cytoperm™, BD Biosciences, San Jose, CA), and set to incubate for 20 minutes in the dark at 4° C. Following incubation, cells were washed in 1mL of Perm Wash Buffer (BD Perm/Wash™, BD Biosciences, San Jose, CA), followed by centrifugation and aspiration, and the addition of 50 µL of Perm Wash Buffer. Intracellular staining for preparation 2 was

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accomplished by adding 1 μL of directly conjugated DyLight® 488-anti-GR to the cell

suspension followed by incubation in the dark for 30 minutes at 4° C. Cells were then washed in

1.0mL of Perm Wash Buffer and resuspended in 1.0mL of stain buffer for flow cytometry

analysis following centrifugation and aspiration.

Flow Cytometry

Flow cytometry analysis of stained cells was performed on a BD Accuri C6 flow

cytometer (BD Biosciences), equipped with BD Accuri analysis software (BD Biosciences).

Forward- and side-scatter, along with four fluorescent channels of data, were collected using two

lasers, providing excitation at 488 nm and 640 nm. A minimum of 10,000 events, defined as

CD14$^+$ monocytes, were obtained with each sample. Monocytes were determined as CD14$^+$

events. Compensation for fluorescence spillover was achieved through single staining of anti-

mouse Ig, κ/negative control compensation particles (BD CompBeads, BD Biosciences).

Unstained leukocytes from human peripheral blood taken at baseline was used as a negative

control for CD14, CD16, CD120a, CD11b, and CCR2 expression, while an IgG isotype control

was used to correct for non-specific binding for glucocorticoid receptor.

Gating Procedures

The gating protocol for preparation 1 is depicted in Figure 3. Viable cells were obtained

using forward-scatter height (FSC-H) x forward-scatter area (FSC-A) gating to eliminate debris,

necrotic cells and artifact (Figure 3: Panels 1 and 2). Following this, monocytes sub-populations

were determined via 2-dimensional histogram (quadrant analysis) based upon CD14 and CD16

expression (Tallone et al., 2011). The quadrant gate was set relative to the unstained control

sample (Figure 3: Panel 3). Analysis of target receptor expression was completed on classical

$CD14^+CD16^-$ monocytes (Figure 3: Panel 4; lower right quadrant) using one dimensional

histograms. An unstained control sample was used to set the gate for all target receptors (Figure

3: Panel 5). The mean fluorescence intensity for each target receptor on $CD14^+CD16^-$ monocytes,

which represents the mean density of each receptor per cell, was quantified by overlaying the

histogram plots of target receptors to the control samples (Figure 3: Panel 6).
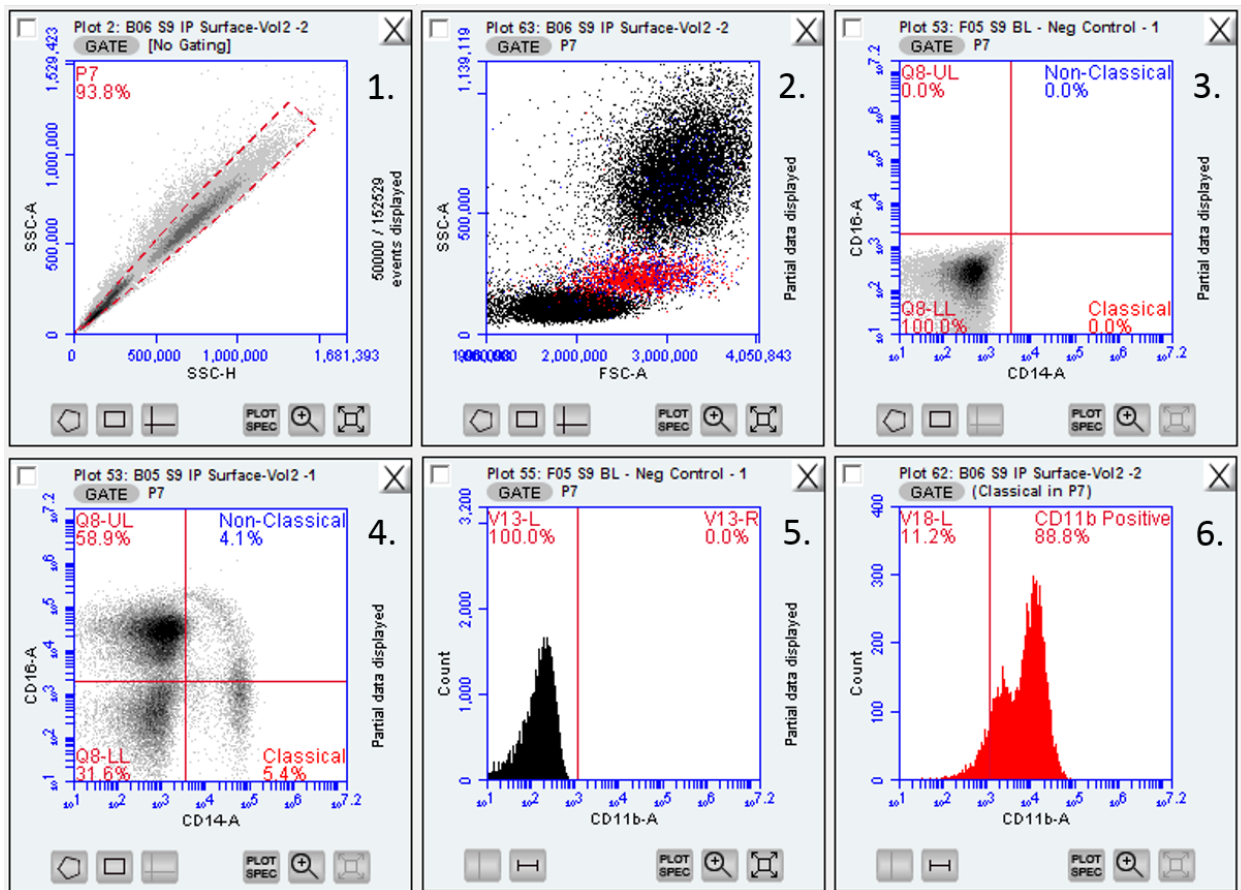
Figure 7. Gating Protocol for Preparation 1

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The gating protocol for preparation 2 is depicted in Figure 4. Viable cells were obtained using forward-scatter height (FSC-H) x forward-scatter area (FSC-A) gating to eliminate debris, necrotic cells and artifact (Figure 4: Panels 1 and 2). Monocytes were determined via one-dimensional histogram analysis of CD14+ cells relative to unstained control (Figure 4: Panels 3 and 4). CCR2 expression on CD14+ monocytes was also quantified via one-dimensional histogram analysis relative to unstained control (Figure 4: Panels 5 and 6). Analysis of glucocorticoid receptor expression was assessed on CD14+/CCR2+ cells (corresponding to classical monocytes) relative to IgG isotype control (Figure 4: Panels 7 and 8). The mean fluorescence intensity for each target receptor was quantified by overlaying the histogram plots to their respective control samples.
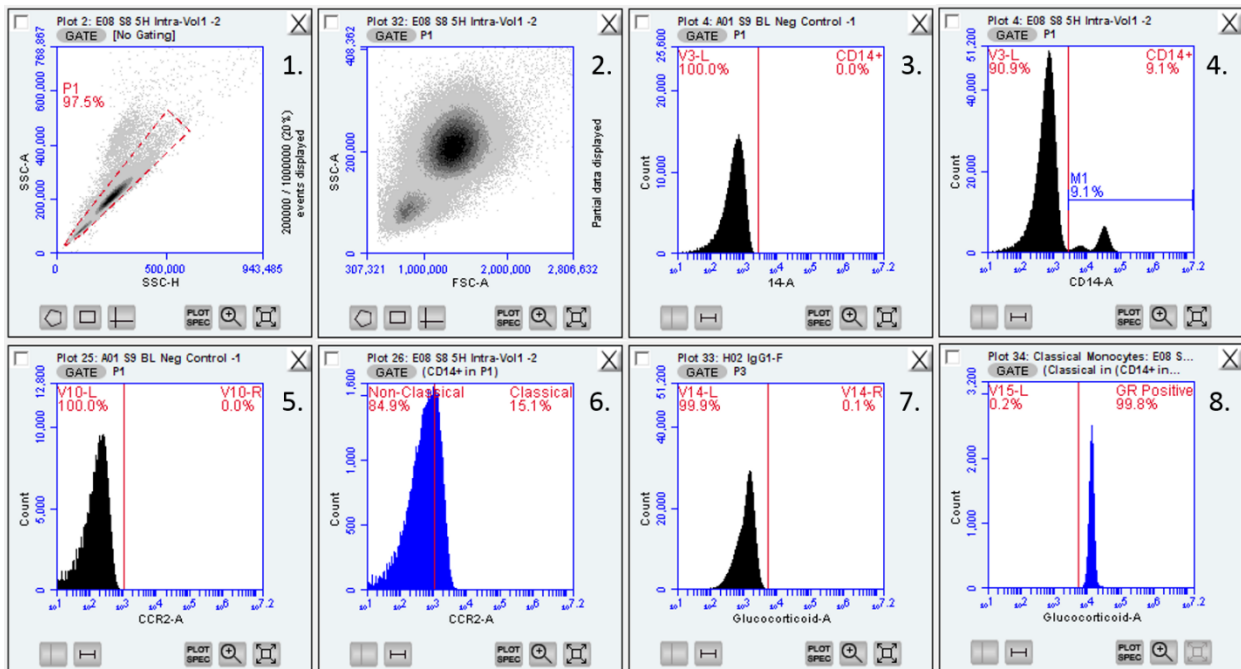

Figure 8. Gating Protocol for Preparation 2

Biochemical and receptor expression changes were analyzed using a two factor (time x trial) repeated measures analysis of variance (ANOVA). In the event of a significant F ratio, dependent t-tests were used for pairwise comparisons between trials. Follow-up one way repeated measures ANOVA were used to determine time effects for each treatment. In the event of a significant F ratio, least significant difference (LSD) post-hoc tests were used for pairwise comparisons across time. Comparisons between trials were further analyzed using Cohen's d. Consistent with others (Clemson et al., 2012), interpretations of Cohen's d were evaluated in accordance with Thalheimer and Cook (2002) at the following levels: negligible effect ($\geq -.15$ and $< .15$), small effect ($\geq = .15$ and $< .40$), medium/moderate effect ($\geq = .40$ and $< .75$), large effect ($\geq = .75$ and <1.10), very large effect ($\geq = 1.10$ and <1.45), and huge effect ≥ 1.45). Time effects were further analyzed using partial eta squared (η^2_p). Interpretations of η^2_p were evaluated in accordance with Cohen (1988) at the following levels: small effect (0.01-0.058), medium effect (0.059-0.137) and large effect (> 0.138). The net area under the curve (AUC) was also calculated for biochemical measures and receptor expression using a standard trapezoidal technique. AUC analyses were performed across either IP, 30P, 1H, 2H and 5H time points (biochemical measures), or IP, 1H, 2H and 5H time points (receptor expression), and were assessed using paired samples t-tests. AUC was used as a proxy to for total receptor expression over time. Pearson's product-moment correlations were used to examine the association between circulating hormones, cytokines and cellular receptor expression on $CD14^{++}CD16^{-}$ monocytes. Absolute values of r were interpreted according to the recommendations of Evans (1996), at the following levels: very weak correlation (.00 to .19), weak correlation (.20 to .39), moderate correlation (.40 to .59), strong correlation (.60 to .79), and very strong correlation (.80 to 1.0).

Correlations between the AUC of circulating measures and the AUC of receptor expression were assessed at IP, 1H, 2H and 5H time points for consistency. Prior to statistical procedures, all data was assessed for sphericity. If the assumption of sphericity was violated, a Greenhouse-Geisser correction was applied. Significance was accepted at an alpha level of $p \leq 0.05$ and all data are reported as mean $\pm$ SD.

CHAPTER IV: FINDINGS

Resistance Exercise Protocol

Differences in workout volume between trials are presented in Figure 9. Workout volume (sets x load x reps) was significantly greater for VOL (45300.0 ± 13919.8 kg) compared to HVY (33633.5 ± 5661.9 kg) (+35%; $d = 1.16$; $p = 0.005$).
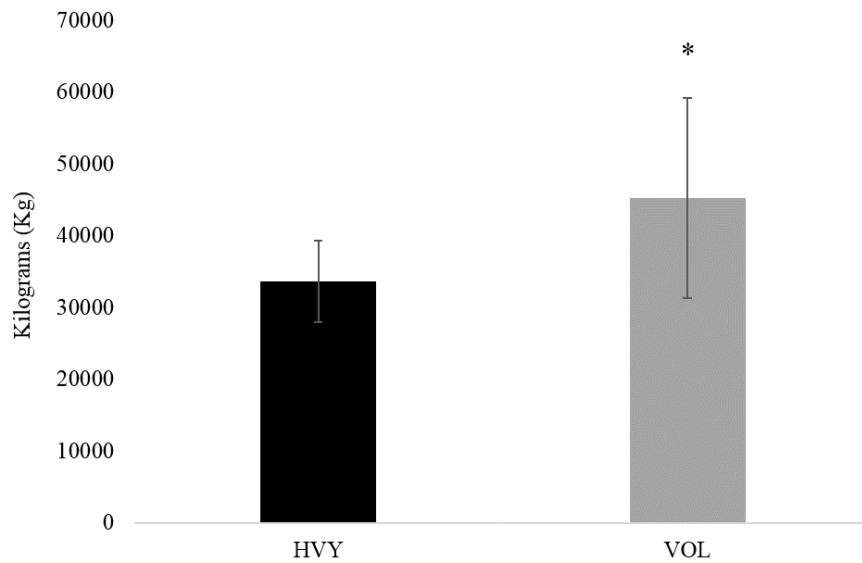

Figure 9. Workout Volume.
Groups: *HVY* = Heavy; *VOL* = Volume. * = Significantly greater volume (p ≤ 0.05); Data reported as means ± SD.

Biochemical Analysis

Lactate

Changes in plasma lactate concentrations are depicted in Table 2. A significant interaction between trials was observed for plasma lactate concentrations (F = 41.7; $p = < 0.001$; $\eta^2_p = 0.82$). Plasma lactate was significantly greater during VOL at IP (+106%; $d = 2.82$; $p = < 0.001$), 30P (+105%; $d = 2.10$; $p = < 0.001$) and 1H (+71%; $d = 1.77$; $p = < 0.001$) compared to HVY. Significant time effects were observed for both HVY (F = 31.8; $\eta^2_p = 0.78$; $p = < 0.001$) and VOL (F = 133.4; $\eta^2_p = 0.94$; $p = < 0.001$). During HVY, lactate was significantly elevated at IP (+396%; $d = 3.1$; $p = < 0.001$), 30P (+176%; $d = 1.82$; $p = 0.004$), 1H (+87%; $d = 1.59$; $p = 0.010$) and 5H (+95%; $d = 1.55$; $p = 0.007$). During VOL, lactate was significantly elevated at IP (+818%; $d = 6.56$; $p = 0.001$), 30P (+409%; $d = 4.48$; $p = < 0.001$), 1H (+187%; $d = 3.75$; $p = < 0.001$), 2H (+73%; $d = 1.32$; $p = 0.002$) and 5H (+93%; $d = 1.36$; $p = 0.009$). AUC was significantly greater for VOL compared to HVY (+51%; $d = 1.28$; $p = 0.009$).

Table 2. Lactate concentration following resistance exercise.

	Lactate (mmol)					
Trial	BL	IP	30P	1H	2H	5H
HVY	1.24 ± 0.21	6.12 ± 2.34 ‡	3.41 ± 1.77 ‡	2.31 ± 0.99 ‡	1.81 ± 1.00	2.41 ± 1.11 ‡
VOL	1.38 ± 0.37	12.63 ± 2.53 ‡*	7.01 ± 1.83 ‡*	3.94 ± 0.95 ‡*	2.39 ± 1.08 ‡	2.66 ± 1.36 ‡

Groups: *HVY* = Heavy; *VOL* = Volume. Time points: *BL* = Baseline; *IP* = Immediately-post; *30P* = 30 minutes post; *1H* = One hour post; *2H* = Two hours post; *5H* = Five hours post. * = Significant difference between trials; ‡ = Significant increase relative to BL ($p \leq 0.05$); Data reported as means ± SD.

Myoglobin

Changes in circulating myoglobin concentrations are presented in Figure 10. A significant interaction between trials was observed for circulating myoglobin (F = 8.3; η^2_p = 0.48; p = 0.003). Myoglobin was significantly greater during HVY at IP (+79%; d = 1.11; p = 0.022), and 30P (+92%; d = 1.28; p = 0.009) compared to VOL. A trend towards HVY being greater than VOL at 1H was observed (+41%; d = 0.74; p = 0.054). Significant time effects were observed for both HVY (F = 21.5; η^2_p = 0.71; p = < 0.001) and VOL (F = 26.3; η^2_p = 0.75; p = < 0.001). During HVY, myoglobin was significantly elevated at IP (+461%; d = 2.14; p = 0.001), 30P (+588%; d = 2.40; p = 0.001), 1H (+582%; d = 2.43; p = 0.001), 2H (+445%; d = 2.30; p = 0.001), and 5H (+285%; d = 2.32; p = 0.001). During VOL, myoglobin was significantly elevated IP (+162%; d = 2.89; p = < 0.001), 30P (+200%; d = 2.81; p = < 0.001), 1H (+305%; d = 3.02; p = < 0.001), 2H (+303%; d = 2.86; p = < 0.001), and 5H (+ 164%; d = 2.47; p = 0.001). Myoglobin AUC is presented in Figure 11. AUC analysis revealed no significant differences between trials (d = 0.60; p = 0.111).

Figure 10. Myoglobin concentration following resistance exercise.
Groups: *HVY* = Heavy; *VOL* = Volume. Time points: *BL* = Baseline; *IP* = Immediately-post;
30P = 30 minutes post; *1H* = One hour post; *2H* = Two hours post; *5H* = Five hours post. * =
Significant difference between trials; ‡ = Significant increase relative to BL ($p \leq 0.05$); Data
reported as means ± SD.
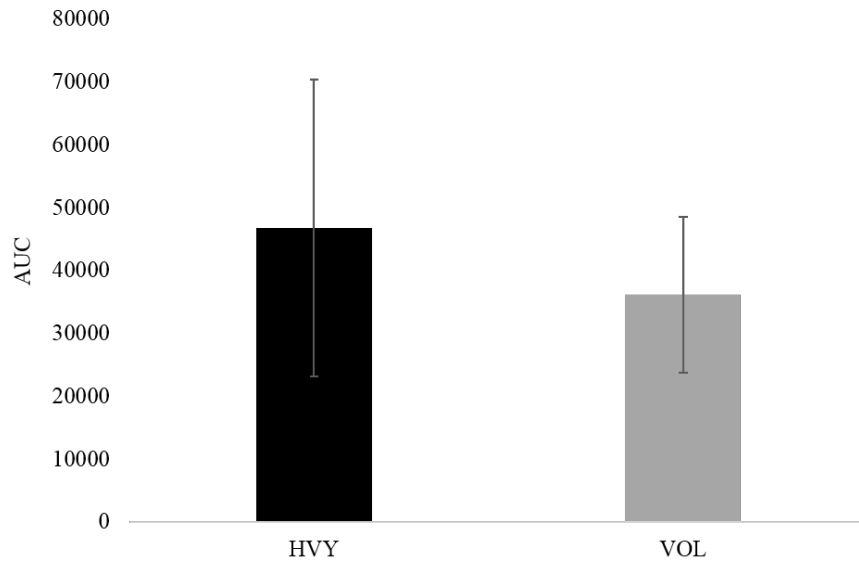
Figure 11. Myoglobin area under the curve (AUC).
Groups: *HVY* = Heavy; *VOL* = Volume. Data reported as means ± SD.

Lactate Dehydrogenase

Changes in circulating LDH concentrations are presented in Figure 12. No significant interactions were noted between trials for circulating LDH (F = 1.3; η^2_p = 0.16; p = 0.283). However, a significant main effect was observed (F = 10.2; η^2_p = 0.59; p = < .001). When collapsed across groups, LDH was significantly elevated above baseline at IP (+34%; d = 1.13; p = 0.006), 30P (+22%; d = 0.78; p = 0.004), 1H (+24%; d = 0.92; p = 0.004), 2H (+33%; d = 1.17; p = 0.001) and 5H (+1.47; d = 1.47; p = <0.001). LDH AUC is presented in Figure 13. AUC was significantly greater for HVY compared to VOL (+11%; d = 0.49; p = 0.010).
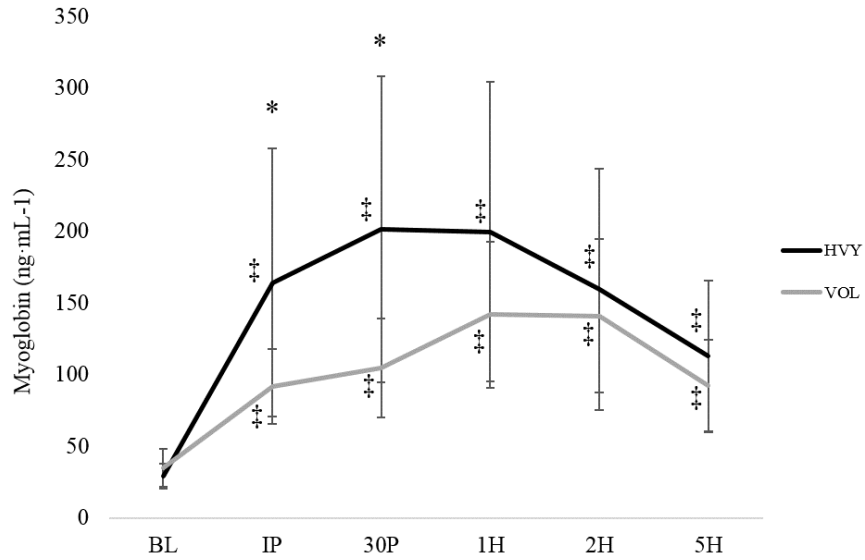

Figure 12. Lactate dehydrogenase (LDH) concentration following resistance exercise. Groups: *HVY* = Heavy; *VOL* = Volume. Time points: *BL* = Baseline; *IP* = Immediately-post; *30P* = 30 minutes post; *1H* = One hour post; *2H* = Two hours post; *5H* = Five hours post. ‡ = Significant increase relative to BL ($p \leq 0.05$); Data reported as means ± SD.
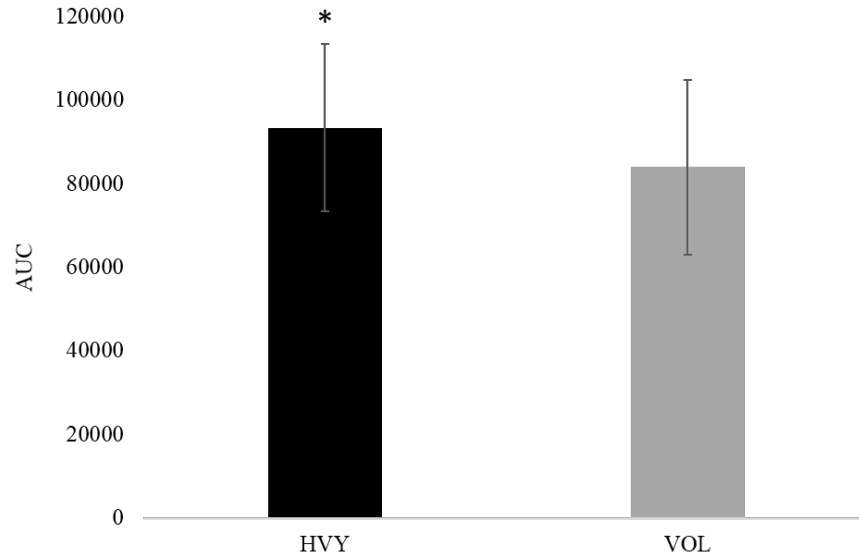
Figure 13. Lactate dehydrogenase (LDH) area under the curve (AUC).
Groups: *HVY* = Heavy; *VOL* = Volume. * = Significantly greater AUC ($p \leq 0.05$); Data reported as means ± SD.

TNFα

Changes in circulating TNFα concentrations are presented in Figure 20. No significant interactions were noted between trials for circulating TNFα (F = 1.7; η^2_p = 0.16; p = 0.218). However, a significant main effect was observed (F = 12.7; η^2_p = 0.56; p = < 0.003). When collapsed across groups, TNFα was significantly elevated at IP (+53%; d = 1.65; p = 0.001), 30P (+66%; d = 1.60; p = 0.002), 1H (+66%; d = 1.56; p = 0.002) and 2H (+33%; d = 1.18; p = 0.004). TNFα AUC is presented in Figure 21. AUC analysis revealed no significant differences between trials (d = 0.31; p = 0.263).

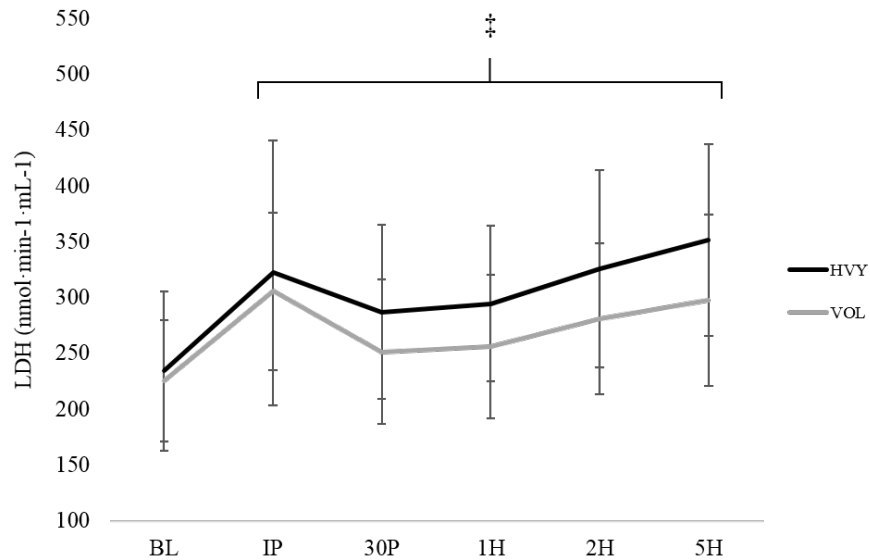Figure 14. Tumor Necrosis Factor - alpha (TNFα) concentration following resistance exercise. Groups: *HVY* = Heavy; *VOL* = Volume. Time points: *BL* = Baseline; *IP* = Immediately-post; *30P* = 30 minutes post; *1H* = One hour post; *2H* = Two hours post; *5H* = Five hours post. ‡ = Significant increase relative to BL ($p \leq 0.05$). Data reported as means ± SD.
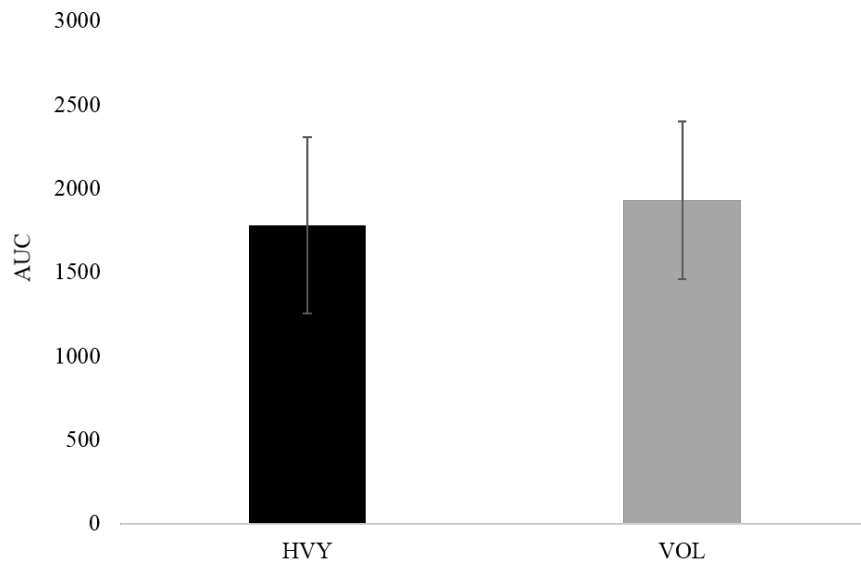
Figure 15. Tumor Necrosis Factor - alpha (TNFα) area under the curve (AUC). Groups: *HVY* = Heavy; *VOL* = Volume. Data reported as means ± SD.

Changes in circulating MCP-1 concentrations are presented in Figure 18. No significant

interactions were observed between trials for circulating MCP-1, although a trend was noted (F =

2.2; η^2_p = 0.19; p = 0.074). However, a significant main effect was observed (F = 6.4; η^2_p = 0.42;

p = < 0.001). When collapsed across groups, MCP-1 was significantly elevated at IP (+76%; d =

1.56; p = 0.005), 30P (+110%; d = 2.02; p = 0.002), 1H (+110%; d = 1.77; p = 0.003), 2H

(+43%; d = 1.21; p = 0.006) and 5H (+37%; d = 0.90; p = 0.033). MCP-1 AUC is presented in

Figure 19. AUC analysis revealed no significant differences between trials (d = 0.20; p = 0.592).
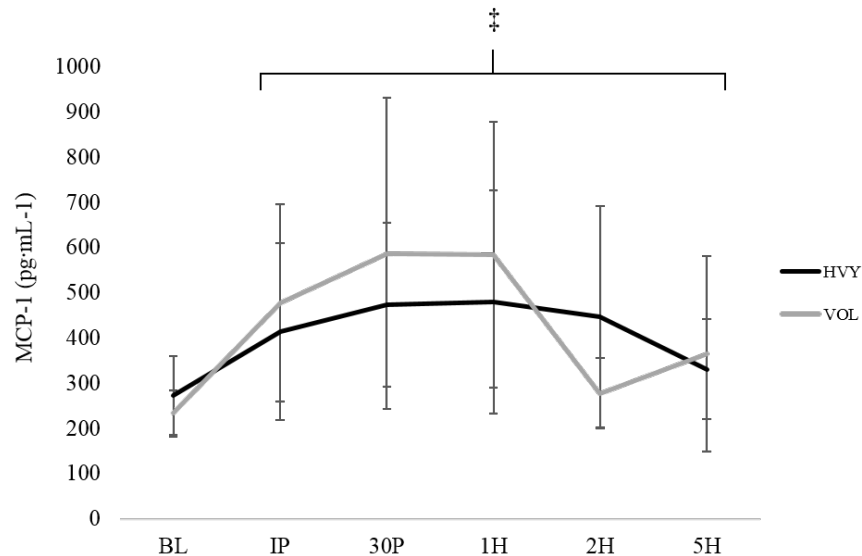
Figure 16. Monocyte chemoattractant protein-1 (MCP-1) concentration following resistance
exercise.
Groups: *HVY* = Heavy; *VOL* = Volume. Time points: *BL* = Baseline; *IP* = Immediately-post;
30P = 30 minutes post; *1H* = One hour post; *2H* = Two hours post; *5H* = Five hours post. ‡ =
Significant increase relative to BL ($p \leq 0.05$). Data reported as means ± SD.
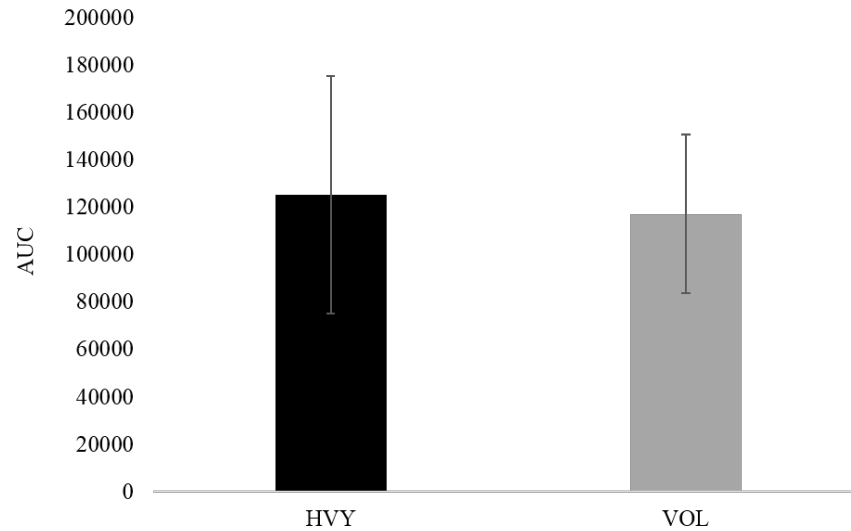
Figure 17. Monocyte Chemoattractant Protein - 1 (MCP-1) area under the curve (AUC). Groups: HVY = Heavy; VOL = Volume. Data reported as means ± SD.

Cortisol

Changes in circulating cortisol concentrations are presented in Figure 16. A significant interaction between trials was observed for cortisol (F = 10.7; η^2_p = 0.54; p = < 0.001). Cortisol was significantly greater during VOL at IP (+57%; d = 1.08; p = 0.012), 30P (+113%, d = 2.12; p = < 0.001), 1H (+91%; d = 2.00; p = 0.003) and 2H (+57%; d = 1.19p; p = 0.018), compared to HVY. Significant time effects were also observed for both HVY (F = 8.9; η^2_p = 0.50; p = < 0.001) and VOL (F = 23.5; η^2_p = 0.72; p = < 0.001). During HVY, cortisol was significantly decreased at 2H (-30%; d = 0.91; p = 0.028) and 5H (-53%; d = 1.87; p = < 0.001) compared to BL. During VOL, cortisol was significantly elevated at IP (+95%; d = 1.40; p = 0.001), 30P (+112%; d = 1.87; p = 0.001), and 1H (+81%; d = 1.51; p = 0.004) compared to BL, and was significantly lower than BL at 5H (-48%; d = 1.07; p = <0.001). Cortisol AUC is presented in

Figure 17. AUC was significantly greater for VOL compared to HVY (+62%; $d = 1.51$; $p = 0.003$).

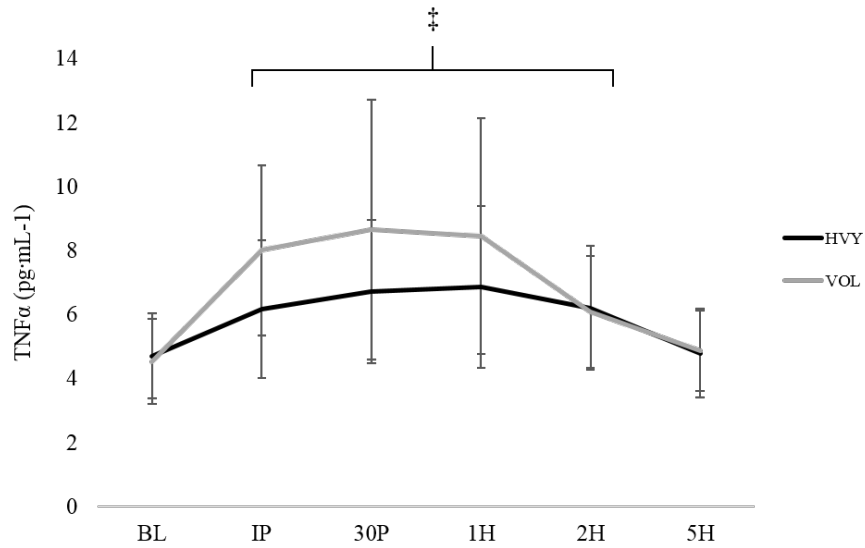Figure 18. Cortisol concentration following resistance exercise.
Groups: *HVY* = Heavy; *VOL* = Volume. Time points: *BL* = Baseline; *IP* = Immediately-post; *30P* = 30 minutes post; *1H* = One hour post; *2H* = Two hours post; *5H* = Five hours post. * = Significant difference between trials; ‡ = Significant increase relative to BL ($p \leq 0.05$); § = Significant decrease relative to BL ($p \leq 0.05$). Data reported as means ± SD.
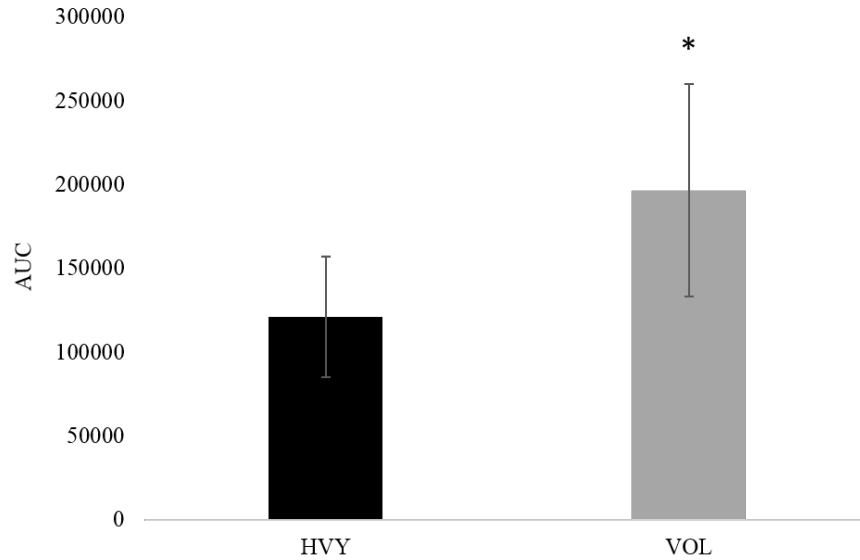
Figure 19. Cortisol area under the curve (AUC).
Groups: *HVY* = Heavy; *VOL* = Volume. * = Significantly greater AUC ($p \leq 0.05$); Data reported as means ± SD.

Testosterone

Changes in circulating testosterone concentrations are presented in Figure 14. A trend was observed between trials for circulating testosterone (F = 2.8; η^2_p = 0.24; p = 0.080). Additionally, a trend towards a main effect was observed for circulating testosterone (F = 3.5; η^2_p = 0.28; p = 0.075). However, neither reached significance. Testosterone AUC is presented in Figure 15. AUC analysis revealed no significant differences between trials (d = 0.02; p = 0.768).
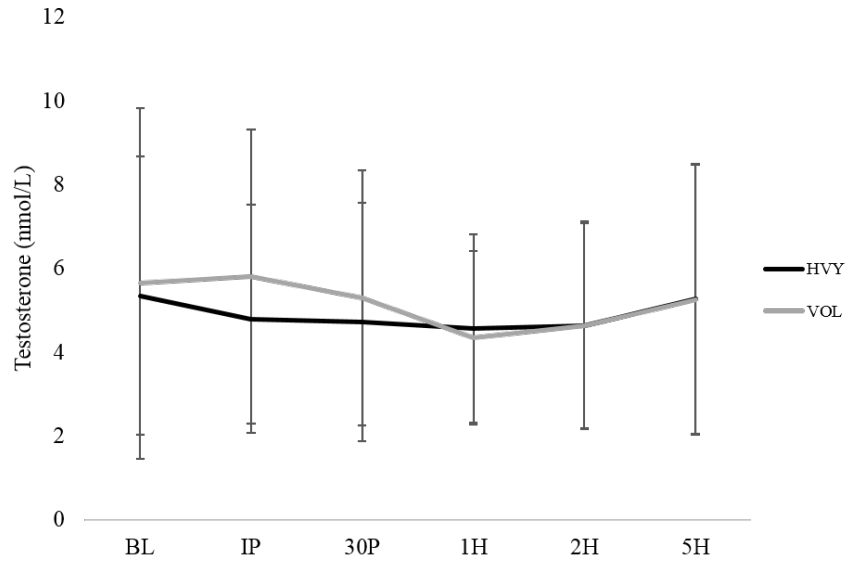
Figure 20. Testosterone concentration following resistance exercise.
Groups: *HVY* = Heavy; *VOL* = Volume. Time points: *BL* = Baseline; *IP* = Immediately-post;
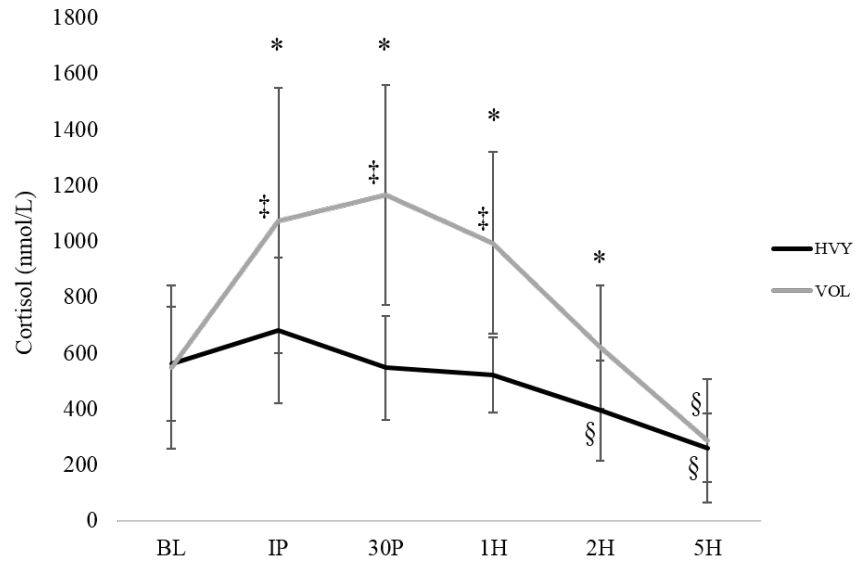*30P* = 30 minutes post; *1H* = One hour post; *2H* = Two hours post; *5H* = Five hours post. Data
reported as means ± SD.
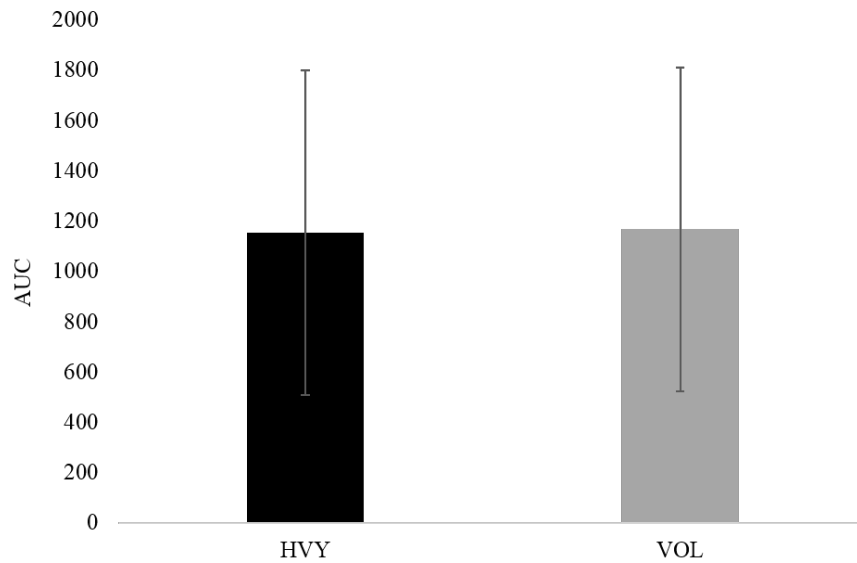
Figure 21. Testosterone area under the curve (AUC).
Groups: *HVY* = Heavy; *VOL* = Volume. Data reported as means ± SD.

<u>Plasma Volume Shifts</u>

Relative to BL, plasma volume shifts were significantly different between trials at IP ($p =$ 0.016). The difference between trials was not significant for any other time-point. During VOL, plasma volume decreased at IP, -8.0 ± 7.7; increased at 30P, 2.1 ± 9.4; increased at 1H, 7.2 ± 14.0; increased at 2H, 3.7 ± 5.0; and decreased at 5H, -1.6 ± 5.5. During HVY, plasma volume decreased at IP, -1.6 ± 3.1; increased at 30P, 3.3 ± 3.6; increased at 1H, 4.0 ± 3.0; increased at 2H, 7.2 ± 7.3; and decreased at 5H, -2.6 ± 4.0. Blood variables were not corrected for plasma volume shifts due to the importance of molar exposure at the cell receptor level.

<u>Flow Cytometry</u>

TNFα Receptor 1 Expression

Changes in TNFr1 expression are presented in Figure 24. No significant interactions were observed between trials for TNFαR1 expression on CD14^{++}CD16$^-$ monocytes (F = 1.0; $\eta^2{}_p =$ 0.10; $p =$ 0.443). In addition, no significant main effect for time was observed (F = 1.2; $\eta^2{}_p =$ 0.12; $p =$ 0.318). TNFr1 AUC is presented in Figure 25. AUC analysis revealed no significant differences between trials ($d =$ 0.21; $p =$ 0.370).

Figure 22. Tumor Necrosis Factor Receptor-1 (TNFr1) expression following resistance exercise. Groups: *HVY* = Heavy; *VOL* = Volume. Time points: BL = Baseline; IP = Immediately-post; 1H = One hour post; 2H = Two hours post; 5H = Five hours post. Data reported as means ± SD.
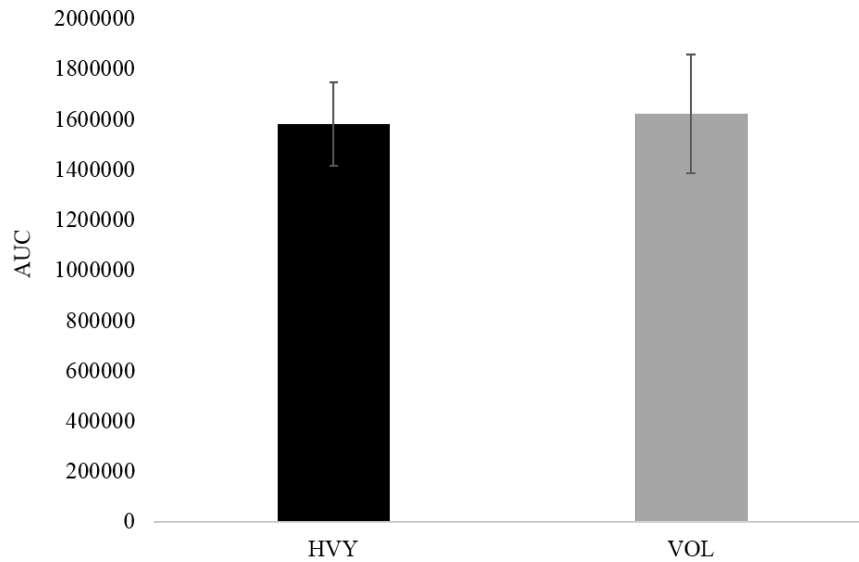
Figure 23. Tumor Necrosis Factor Receptor-1 (TNFr1) area under the curve (AUC). Groups: *HVY* = Heavy; *VOL* = Volume. Data reported as means ± SD.

Changes in CCR2 expression are presented in Figure 22. No significant interactions were observed between trials for CCR2 expression on $CD14^{++}CD16^{-}$ monocytes (F = 0.4; p = 0.696; η^2 = 0.04). However, a significant main effect was observed (F = 4.6; p = 0.005; η^2 = 0.37). When collapsed across groups, CCR2 expression on $CD14^{++}CD16^{-}$ monocytes was significantly lower at IP (-25%; d = 1.32; p = 0.033), 1H (-25%; d = 1.37; p = 0.020), 2H (-28%; d = 1.32; p = 0.040) and 5H (-29%; 1.43; p = 0.024). CCR2 AUC is presented in Figure 23. AUC analysis revealed a trend towards a significant difference between trials (d = 0.73; p = 0.056).
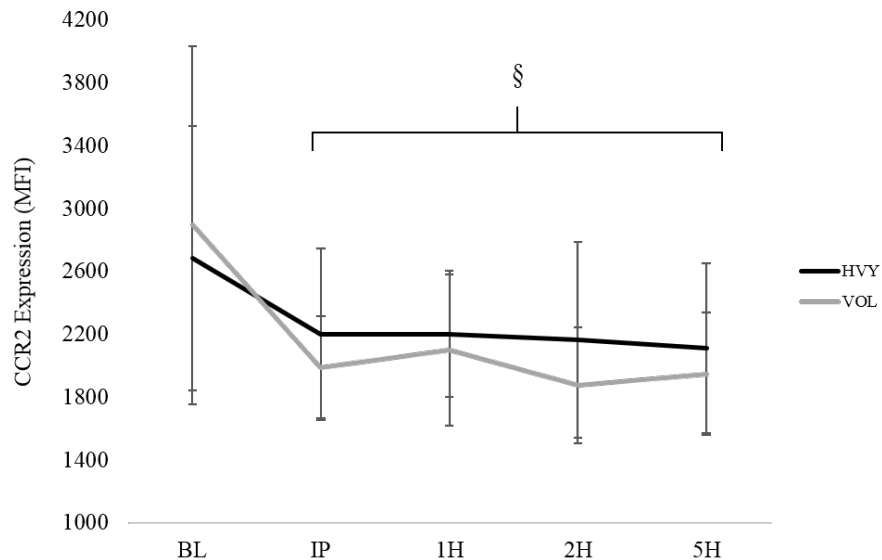
Figure 24. C-C Chemokine Receptor 2 expression following resistance exercise.
Groups: *HVY* = Heavy; *VOL* = Volume. Time points: *BL* = Baseline; *IP* = Immediately-post; *1H* = One hour post; *2H* = Two hours post; *5H* = Five hours post. § = Significant decrease relative to BL ($p \leq 0.05$). Data reported as means ± SD.
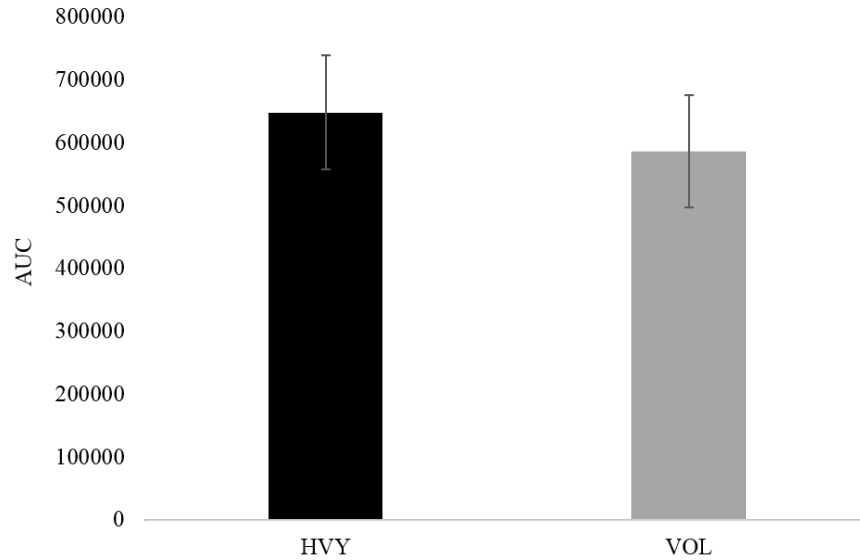
Figure 25. C-C Chemokine Receptor 2 area under the curve (AUC).
Groups: *HVY* = Heavy; *VOL* = Volume. Data reported as means ± SD.

Glucocorticoid Receptor Expression

Changes in GCR expression are presented in Figure 28. No significant

interactions were noted between trials for Glucocorticoid receptor expression on CD14^{++}CD16^{-}

monocytes (F = 0.9; p = 0.460; η^2_p = 0.12). A significant time effect was observed (F = 4.0; p =

0.011; η^2_p = 0.36). However; when collapsed across groups, no significant elevations above

baseline were observed (p = > 0.131). GCR AUC is presented in Figure 29. AUC analysis

revealed no significant differences between trials (d = 0.30; p = 0.505).
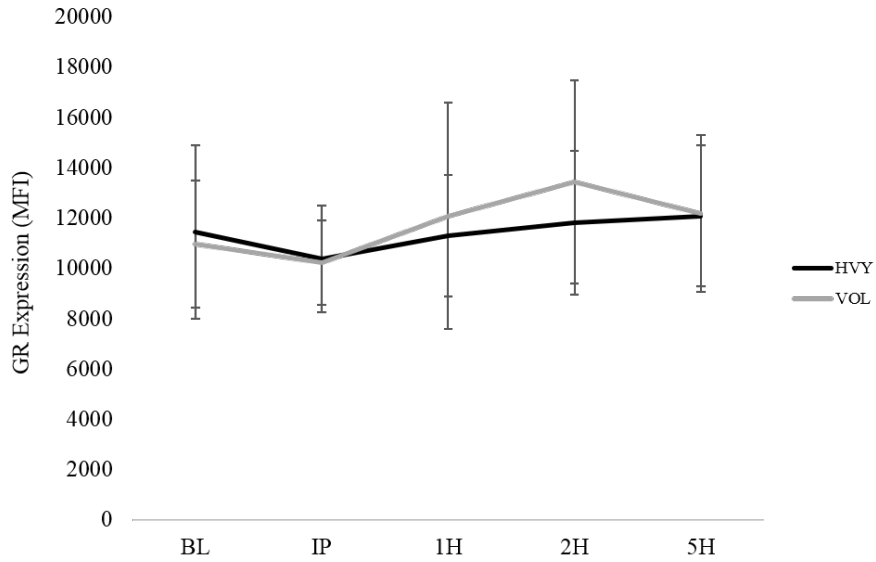
Figure 26. Glucocorticoid receptor expression following resistance exercise.
Groups: *HVY* = Heavy; *VOL* = Volume. Time points: *BL* = Baseline; *IP* = Immediately-post;
1H = One hour post; *2H* = Two hours post; *5H* = Five hours post. Data reported as means ± SD.
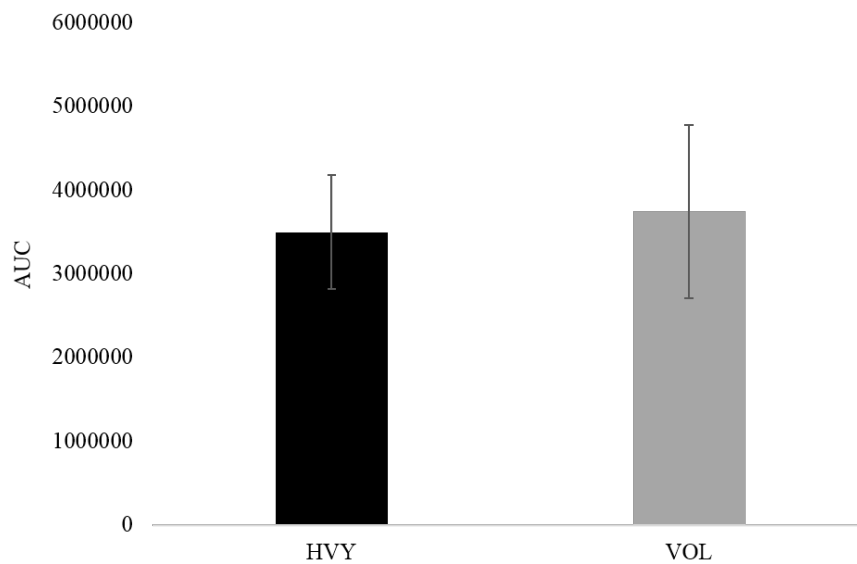
Figure 27. Glucocorticoid receptor area under the curve (AUC).
Groups: *HVY* = Heavy; *VOL* = Volume. Data reported as means ± SD.

CD11b Receptor Expression

Changes in CD11b receptor expression are presented in Figure 26. No significant interactions were observed between trials for CD11b expression on CD14^{++}CD16$^-$ monocytes (F = 0.7; p = 0.599; η^2_p = 0.07). However, a significant time effect was observed (F = 6.0; p = 0.001; η^2_p = 0.40). When collapsed across groups, CD11b receptor expression on CD14^{++}CD16$^-$ monocytes was significantly greater at 1H (+28%; d = 0.71; p = 0.001). A trend towards an increase in CD11b receptor expression was observed at IP (+21%; d = 0.55; p = 0.067). Additionally, a trend towards a decrease in CD11b receptor expression was observed at 5H (-17%; d = 0.50; p = 0.082). CD11b AUC is presented in Figure 27. AUC analysis revealed no significant differences between trials (d = 0.04; p = 0.894).
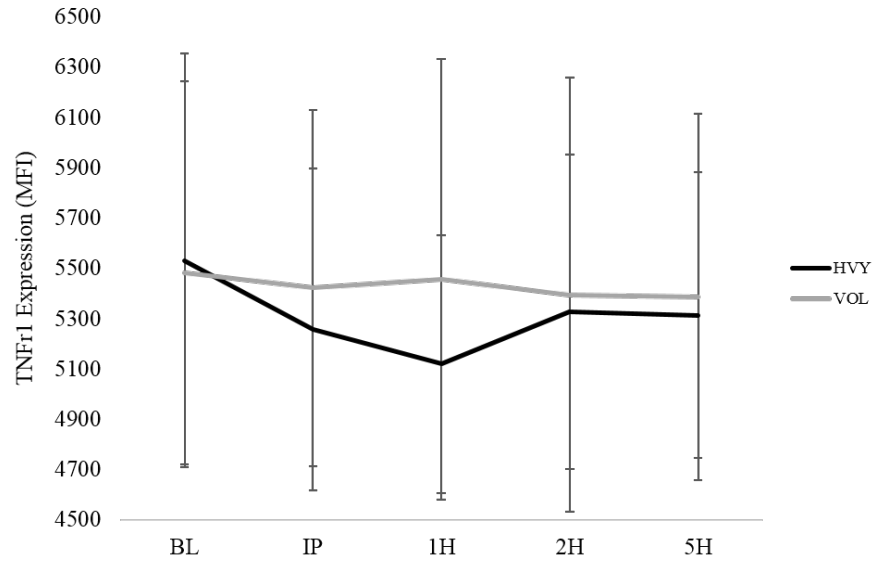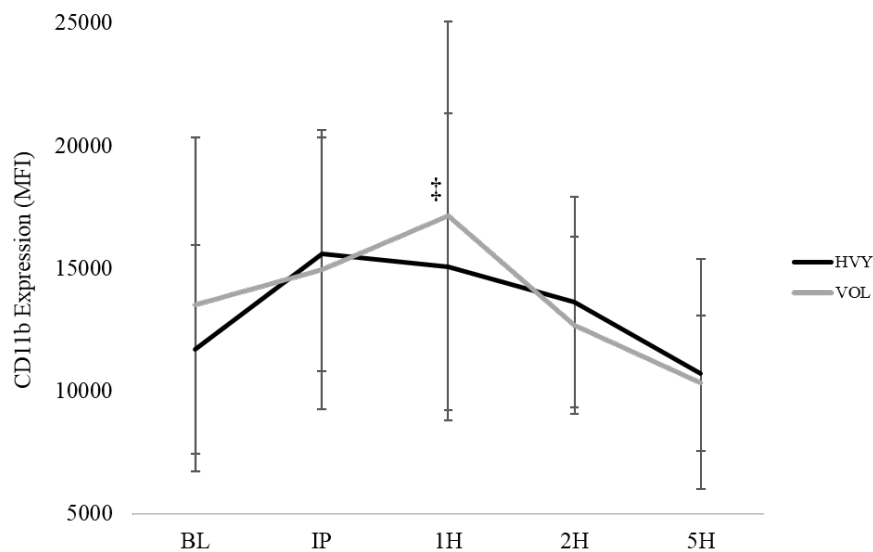

Figure 28. CD11b expression following resistance exercise.
Groups: *HVY* = Heavy; *VOL* = Volume. Time points: *BL* = Baseline; *IP* = Immediately-post; *1H* = One hour post; *2H* = Two hours post; *5H* = Five hours post. ‡ = Significant increase relative to BL ($p \leq 0.05$). Data reported as means ± SD.
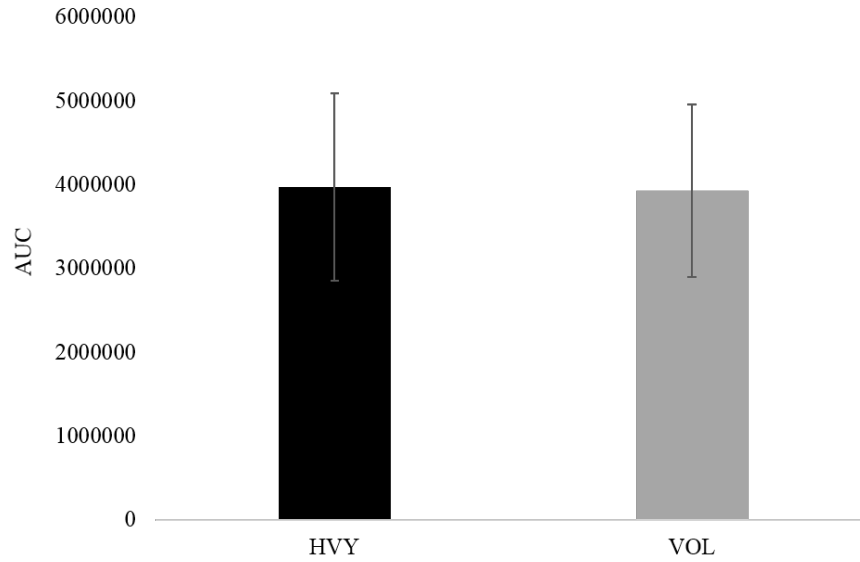
Figure 29. CD11b area under the curve (AUC).
Groups: *HVY* = Heavy; *VOL* = Volume. Data reported as means ± SD.

<u>Correlations</u>

CD11b receptor expression was positively correlated with circulating MCP-1 at 1H (r = .632, p = 0.003), and with circulating TNFα at 1H (r = .552, p = 0.012).

CHAPTER V: DISCUSSION

The findings of this study indicate that changes in CCR2, CD11b, and GCR expression on $CD14^{++}CD16^{-}$ monocytes are similar following acute bouts of high-intensity, and high-volume resistance exercise. When collapsed across groups, CCR2 expression was significantly lower at IP, 1H, 2H and 5H post-exercise, while CD11b expression was significantly elevated at 1H post-exercise only. GCR expression was not significantly different from baseline at any time point. Plasma concentrations of myoglobin were significantly higher following HVY compared to VOL; however, both protocols resulted in significant elevations above BL at all post-exercise time points. No difference between trials was observed for LDH, however AUC was significantly greater in HVY compared to VOL. Changes in plasma TNFα, and MCP-1 concentrations were similar between HVY and VOL. As expected, significant elevations in plasma cortisol concentrations were observed following VOL only, although this did not appear to be related to changes in receptor expression. Plasma testosterone concentrations and TNFr1 receptor expression did not appear to be affected by resistance exercise.

The propagation of muscle damage results in an inflammatory response that is characterized by the production of pro-inflammatory cytokines such as TNFα. Our results indicate that plasma TNFα is rapidly increased following resistance exercise. Relative to BL, plasma TNFα was significantly increased at IP, 30P, 1H and 2H post-exercise, and this was similar between HVY and VOL. This suggests that resistance exercise, irrespective of HVY or VOL, resulted in a robust post-exercise inflammatory response. This is consistent with prior work from our laboratory (Townsend et al., 2015). We have previously reported significant increases in plasma TNFα immediately, and 30 minutes post-exercise, following an acute bout of high-volume resistance exercise in well-trained males. In the present study, we further

characterize the TNFα response to damaging resistance exercise, demonstrating that plasma concentrations remain elevated for up to 2 hours post-exercise. In contrast to our findings, a number of studies report no changes in plasma TNFα following exercise. Brenner et al. (1999) found no changes in plasma TNFα concentrations following an acute bout of high-volume lower-body resistance exercise in untrained males (Brenner et al., 1999). Similarly, Smith et al. (2000) found no changes in plasma TNFα following high-volume eccentric resistance exercise at 100% 1RM in untrained males. Further, Peake et al. (2006) found no changes in plasma TNFα concentrations following submaximal and maximal lengthening contractions of the elbow flexors in untrained males (Peake, Nosaka, Muthalib, & Suzuki, 2006). Differences between the present study and these other investigations suggest that the cytokine response may differ between trained and untrained individuals. There is evidence to suggest that the leukocyte and cytokine response may be delayed following significant tissue damage (Paulsen et al., 2005; Suzuki et al., 2002). In untrained individuals, an exercise stimulus of sufficient magnitude, particularly if unaccustomed, may result in a more pronounced muscle damage compared to trained individuals. This may subsequently lead to a delayed cytokine response, which may explain some of the differences between studies. Notwithstanding, the temporal behavior of cytokines in response to resistance exercise requires further investigation.

TNFα plays several important roles in inflammation, including inducing the secretion of MCP-1 (Murao et al., 2000), up-regulating ICAM-1 and P-selectin adhesion molecules on endothelial cells (Bernot, Peiretti, Canault, Juhan-Vague, & Nalbone, 2005), and up-regulating CD11b expression on both neutrophils and monocytes (Montecucco et al., 2008; Sumagin et al., 2010). These effects are mediated via binding with its cognate membrane receptors TNFr1 (CD120a) and TNFr2 (CD120b) (Hijdra, Vorselaars, Grutters, Claessen, & Rijkers, 2012).

However, TNFr1 appears to be the key mediator of TNFα signaling (Grell, Wajant, Zimmermann, & Scheurich, 1998; Wajant, Pfizenmaier, & Scheurich, 2003). TNFr1 expression did not change significantly in response to HVY or VOL resistance exercise. We have previously reported a significant increase in TNFr1 receptor expression on monocytes following acute bouts of high-volume resistance exercise in well-trained males (Townsend et al., 2013; Townsend et al., 2015). However, in contrast to the present study, this observation was made on CD14^{++} monocytes, without further differentiation based on the expression of CD16 antigen. Classical and intermediate monocytes both express a high level of CD14 antigen (Wong et al., 2011), which prevents their differentiation in the absence of a secondary antibody. Recent research suggests that intermediate monocytes express TNFr1 to a significantly greater extent compared to classical and non-classical monocytes (Hijdra et al., 2012). It is therefore possible that the change in TNFr1 expression previously reported by our laboratory occurred in intermediate CD14^{++} CD16^{+} monocytes rather than CD14^{++}CD16^{-} classical monocytes. Nevertheless, examinations of temporal changes in the expression of TNFr1 on monocytes following resistance exercise are lacking.

Our results indicate that circulating MCP-1 is also rapidly increased following damaging resistance exercise. Under inflammatory conditions, MCP-1 is the primary cytokine involved in mediating classical monocyte chemotaxis (Charo & Ransohoff, 2006; B. Lu et al., 1998; Rossi & Zlotnik, 2000; Shireman et al., 2006; Shireman et al., 2007; M. Takahashi et al., 2009). Relative to baseline, MCP-1 was significantly elevated at all post-exercise time points, and this response was similar between HVY and VOL. This is consistent with the reports indicating that circulating concentrations of MCP-1 parallel early inflammation (Shireman et al., 2006), and

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suggests that both HVY and VOL protocols likely resulted in a significant recruitment of monocytes.

To our knowledge, only one other study has examined changes in plasma MCP-1 in conjunction with an acute bout of resistance exercise (Ihalainen et al., 2014). In contrast to our findings, Ihalainen and colleagues (2014) report a significant decrease in plasma MCP-1 concentrations, following an acute bout of high-volume leg press exercise [5 x 10 (80% 1RM)] in healthy untrained males. Data regarding the effect of training status on the plasma MPC-1 response is currently lacking. However, differences between findings could be related to the magnitude of the exercise stimulus. We utilized an exercise protocol consisting of 5 exercises designed to target the entire lower extremity. In contrast, Ihalainen and colleagues utilized only the leg press exercise. Given the large disparity in total work between studies, it is possible that a minimal exercise volume may be required to stimulate MCP-1, and that this was not attained with the leg press alone. Nevertheless, Ihalainen and colleagues only examined plasma MCP-1 concentrations up to 30 minutes post-exercise. Therefore, a delayed increase in plasma MCP-1 cannot be discounted.

Consistent with our findings, significant changes in plasma MCP-1 have been reported following a number of aerobic exercise interventions (Crystal, Townson, Cook, & LaRoche, 2013; Peake, Suzuki, Hordern et al., 2005). Peake et al. (2005) observed significant increases in plasma MCP-1 immediately, and 1 hour post-exercise, following three separate running protocols of varying intensity (Peake et al., 2005). Similarly, Crystal et al. (2013) observed a significant increase in plasma MCP-1 immediately, 1 hour, and 6 hours post-exercise, following 40 minutes of downhill running at 60% VO_{2max}. Comparisons between aerobic exercise protocols appear to suggest that the MCP-1 response is related to exercise intensity (%VO_{2max}) rather than

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the degree of muscle damage (Peake et al., 2005). This is consistent with reports that plasma

MCP-1 concentrations are higher following level running at 85% VO_{2max}, despite a significantly

lower plasma concentration of myoglobin compared to downhill running (Peake, Suzuki, Wilson

et al., 2005; Peake et al., 2005). Anecdotally, our findings appear to support this notion, since

MCP-1 trended ($p = 0.074$) towards being higher following high-volume resistance exercise

compared to the more damaging high-intensity protocol. Nevertheless, changes in MCP-1 were

not significantly different between trials. Collectively, it appears that exercise of a sufficient

magnitude results in a rapid elevation in plasma MCP-1. This is likely in response to a

disturbance in tissue homeostasis (Soehnlein & Lindbom, 2010), which may or may not be

related to the degree of muscle damage. Further research is required to fully elucidate the effects

of exercise on the magnitude of the MCP-1 response.

MCP-1 exerts its chemotactic function via exclusive binding to the G-protein coupled

receptor CCR2 (Rot & von Andrian, 2004). Our result indicate that CCR2 expression is

significantly down-regulated at all post-exercise time points following both high-intensity and

high-volume resistance exercise. The observed findings may be the result of agonist mediated

receptor internalization, which is a well characterized mechanism contributing to the tight

control of inflammation (Bennett, Fox, & Signoret, 2011; Berchiche, Gravel, Pelletier, St-Onge,

& Heveker, 2011; Kelly, Bailey, & Henderson, 2008; Rot & von Andrian, 2004). However, to

the best of our knowledge, no other study has evaluated changes in CCR2 expression following

an acute bout of resistance exercise.

Changes in CCR2 expression have been reported following examinations of MCP-

1/CCR2 mediated monocyte chemotaxis *in vitro* (Arai, Monteclaro, Tsou, Franci, & Charo,

1997; Franci, Gosling, Tsou, Coughlin, & Charo, 1996; Handel et al., 2008). Handel and

colleagues (2008) observed a dose-dependent decrease in CCR2 expression following incubation of murine monocytes with MCP-1. A substantial down-regulation of CCR2 receptors was apparent within 30 minutes, while one hour of incubation was sufficient to induce a 60% down-regulation of CCR2 receptors. Upon removal of the MCP-1 ligand, CCR2 expression began to recover. Nevertheless, maximal receptor expression was not regained for an additional 4 hours. Consistent with this, low nanomolar concentrations of MCP-1 have been shown to be sufficient to induce a substantial down-regulation of CCR2. Further, this effect was achieved within 5 to 10 minutes of MCP-1 exposure (Franci et al., 1996). Together, these results suggest that MCP-1 may induce the internalization of CCR2 receptors, possibly as part of a mechanism designed to modulate the magnitude of the monocyte response.

A recent investigation by Volpe and colleagues (2012) indicates that internalization of CCR2 does not reduce the responsiveness of monocytes to MCP-1 (Volpe et al., 2012). These investigators suggest that following interaction with MCP-1, internalized receptors rapidly recycle back to the cell surface in order to maintain the responsiveness of the cell towards the chemokine. Physiologically, this action provides for continuous signaling from receptors at the leading edge of the monocyte, which is required for directional migration. This mechanism would allow monocytes to proceed along an increasing chemokine gradient without becoming desensitized. Nevertheless, under conditions of continuous stimulation, receptor cycling may still lead to a decrease in CCR2 receptor expression. However, given the need to maintain cell polarity, it is likely that that this response would be limited to a particular threshold, rather than being dose-dependent. This is consistent with the plateau observed in the present study, and may also explain why we did not find a significant inverse relationship between plasma MCP-1 concentrations and CCR2 expression.

Cortisol has been shown to modulate the expression of CCR2 on human monocytes *in vitro* (Okutsu et al., 2008; Penton-Rol et al., 1999; Pettersson et al., 2005). Despite a significant increase in plasma cortisol following the high-volume protocol, changes in the expression of CCR2 were not different between trials. Additionally, changes in GCR expression were not different from BL, and were similar between the two acute protocols. Consequently, our results do not support the *in vitro* observations of others. It is worth mentioning however, that at least 4-6 hours of incubation were required before an up-regulation of CCR2 was observed *in vitro* (Okutsu et al., 2008; Penton-Rol et al., 1999). Further, 60 minutes of cycling at 70% VO_{2peak} was unable to stimulate an up-regulation of CCR2 on monocytes *in vivo*, even following 24 hours of additional incubation time (Okutsu et al., 2008). In the present study, plasma cortisol concentrations did not correlate with any outcome measure to a physiologically meaningful degree.

CD11b mediates monocyte intravascular crawling, which is the direct pre-requisite to trans-endothelial migration (Ley et al., 2007; Sumagin et al., 2010). Our results indicate a moderate but significant up-regulation in CD11b at 1H post-exercise. Further, this change was similar between high-intensity and high-volume trials. The up-regulation of CD11b at 1H post-exercise indicates a greater potential for monocyte adhesion, and is consistent with the temporal appearance of monocytes at sites of tissue damage reported by McLennan (McLennan, 1996). We have previously reported similar increases in CD11b on CD14^{++} monocytes following acute bouts of high-volume resistance exercise in well-trained males (Gonzalez et al., 2014; Jajtner et al., 2014). Our reports appear to be consistent with the findings of others (Hong & Mills, 2008; Jordan et al., 1999). Hong et al. (2008) observed a significant increase in the expression of CD11b on CD14^{++}CD16^{-} monocytes immediately, and 10-minutes post-exercise, following a 20-

minute bout of moderate treadmill exercise at 65-70% VO_{2peak} (Hong & Mills, 2008). In another study, a significant up-regulation in CD11b expression was observed following maximal treadmill exercise and marathon running, albeit on granulocytes (Jordan et al., 1999). Interestingly, a bout of moderate treadmill exercise was not sufficient to cause an up-regulation in CD11b (Jordan et al., 1999). This could indicate that exercise must be of a sufficient volume and/or intensity for CD11b expression to be increased.

The observed increase in CD11b expression correlated moderately with plasma concentrations of both TNFα, and MCP-1 at 1H. These findings are supported by a number of *in vitro* and *in situ* studies, suggesting that both observations could be physiologically meaningful (Montecucco et al., 2008; Starikova et al., 2010; Sumagin et al., 2010; K. Takahashi et al., 2003; van Royen et al., 2003). However, TNFα and MCP-1 were also highly correlated with each other at all post-exercise time points (data not shown), suggesting that the association of at least one of these ligands with CD11b up-regulation is spurious. Previous work by Campbell et al. (2000) indicates that for a given receptor, the signals required for rapid adhesion to vascular integrin ligands are different from those required for chemotaxis, illustrating that two distinct G-protein-linked receptor-dependent events are required for leucocyte extravasation from the blood (Campbell, Foxman, & Butcher, 1997). This suggests that TNFα mediated up-regulation of CD11b is more likely, since MCP-1 mediates chemotaxis. Nevertheless, we are unable to delineate the contributions of MCP-1 and/or TNFα in the regulation of CD11b expression.

Conclusions

The present study investigated the acute pro-inflammatory response following two typical lower-body resistance exercise protocols in experienced, resistance trained men. Markers of muscle damage were elevated to a significantly greater extent in HVY, while plasma cortisol concentrations were significantly greater following VOL. Nevertheless, changes in plasma concentrations of TNFα, and MCP-1 were similar between HVY and VOL, as was the temporal response of TNFr1, CCR2, GCR and CD11b receptors on CD14^{++} CD16$^-$ monocytes. Consequently, our results do not support a role for cortisol in the modulation of these receptors *in vivo*, while the degree of muscle damage does not appear to influence plasma concentrations of TNFα, or MCP-1. It is therefore likely that both HVY and VOL protocols constitute an exercise stimulus that is sufficient enough to promote a robust pro-inflammatory response, which is similar in timing and magnitude.

APPENDIX A: UCF IRB APPROVAL LETTER

University of Central Florida Institutional Review Board
Office of Research & Commercialization
12201 Research Parkway, Suite 501
Orlando, Florida 32826-3246
Telephone: 407-823-2901, 407-882-2901 or 407-882-2276
www.research.ucf.edu/compliance/irb.html

Notice that UCF will Rely Upon Other IRB for Review and Approval

From : **UCF Institutional Review Board**
FWA00000351, IRB00001138

To : **Adam Michael Gonzalez**

Date : **August 21, 2014**

IRB Number: **SBE-14-10394**

Study Title: **The effect of different heavy resistance exercise protocols and a proprietary blend protein supplement on protein signaling, hormonal, and pro-inflammatory immune response following resistance exercise**

Dear Researcher:

The research protocol noted above was reviewed by the University of Central Florida designated Reviewer on August 21, 2014. The UCF IRB accepts the New England's Institutional Review Board review and approval of this study for the protection of human subjects in research. **The expiration date will be the date assigned by the New England's Institutional Review Board and the consent process will be the process approved by that IRB.**

This project may move forward as described in the protocol. It is understood that the New England's IRB is the IRB of Record for this study, but local issues involving the UCF population should be brought to the attention of the UCF IRB as well for local oversight, if needed.

All data, including signed consent forms if applicable, must be retained and secured per protocol for a minimum of five years (six if HIPAA applies) past the completion of this research. Any links to the identification of participants should be maintained and secured per protocol. Additional requirements may be imposed by your funding agency, your department, or other entities. Access to data is limited to authorized individuals listed as key study personnel.

Failure to provide a continuing review report for renewal of the study to the New England's IRB could lead to study suspension, a loss of funding and/or publication possibilities, or a report of noncompliance to sponsors or funding agencies. If this study is funded by any branch of the Department of Health and Human Services (DHHS), an Office for Human Research Protections (OHRP) IRB Authorization form must be signed by the signatory officials of both institutions and a copy of the form must be kept on file at the IRB office of both institutions.

On behalf of Sophia Dziegielewski, Ph.D., L.C.S.W., UCF IRB Chair, this letter is signed by:

Signature applied by Patria Davis on 08/21/2014 10:10:13 AM EDT

IRB Coordinator

APPENDIX B: NEIRB APPROVAL LETTER

NEW ENGLAND INSTITUTIONAL REVIEW BOARD

August 20, 2014

Adam M. Gonzalez, MEd
University of Central Florida
12494 University Boulevard
Orlando, FL 32816

Re: (IRB# 14-272): "The Effect of Different Heavy Resistance Exercise Protocols and a Proprietary Blend Protein
        Supplement on Protein Signaling, Hormonal, and Pro-Inflammatory Immune Response Following Resistance
        Exercise"

This is to inform you that New England Institutional Review Board (NEIRB)'s Tuesday Board has approved the above-
referenced research protocol and the participation of the above-referenced investigative site in the research. The approval
period is **8/20/2014 to 7/7/2015. Your study number is 14-272. Please be sure to reference either this number or the
name of the principal investigator in any correspondence with NEIRB.**

Continued approval is conditional upon your compliance with the following requirements:
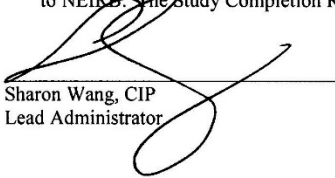
- A copy of the **Informed Consent Document, NEIRB version 1.0**, approved as of 8/20/2014 is enclosed. Only NEIRB-
  approved informed consent documents should be used. It must be signed by each subject prior to initiation of any protocol
  procedures. In addition, each subject must be given a copy of the signed consent form.

- The following must be promptly reported to NEIRB: changes to the study site, and all unanticipated problems that may
  involve risks or affect the safety or welfare of subjects or others, or that may affect the integrity of the research.

- Approval is valid for enrollment of the number of subjects indicated on your submission form. If you anticipate enrolling
  more than this number of subjects, NEIRB approval must be obtained prior to exceeding the approved enrollment number.

- All protocol amendments and changes to approved research must be submitted to the IRB and not be implemented until
  approved by the IRB except where necessary to eliminate apparent immediate hazards to the study subjects.

- Compliance with all federal and state laws pertaining to this research, and with NEIRB's SOPs.

- The enclosed recruitment/subject materials have been approved (Recruitment Script, Dietary Logs and Instructions,
  Confidential Medical and Activity History Questionnaire, Physical Activity Readiness Questionnaire (PAR-Q), Soreness
  Recovery Scale, and Visual Analog Scale). Advertisements, letters, internet postings and any other media for subject
  recruitment must be submitted to NEIRB and approved prior to use. Please refer to *NEIRB Guidelines for Recruitment and
  Advertising*, available at www.neirb.com.

- All deaths, life-threatening problems or serious or unexpected adverse events, *whether related to the study article or not*,
  must be reported to the IRB. The Serious Adverse Event Form is available at www.neirb.com.

- Any and all necessary FDA approvals must be received prior to your initiation of the trial. If this study is being conducted
  under an IDE, a copy of the FDA IDE approval letter must be submitted to NEIRB.

- The study cannot continue after 7/7/2015 until re-approved by NEIRB. A Study Renewal Report must be completed and
  returned to NEIRB prior to the expiration of the approval period.

*Full
AAHRPP
Accreditation*

- When the study is completed, terminated, or if it is not being renewed - complete and submit a Study Completion Report to NEIRB. The Study Completion Report can be accessed via the NEIRB website at www.neirb.com.

Sharon Wang, CIP
Lead Administrator

Copy: NEIRB Chair
          University of Central Florida IRB
          Enclosures

APPENDIX C: INFORMED CONSENT

The effect of different heavy resistance exercise protocols and a proprietary blend protein supplement on protein signaling, hormonal, and pro-inflammatory immune response following resistance exercise

Informed Consent

Principal Investigator:	Adam M. Gonzalez, M.Ed.
Sub-Investigator(s):	Jay R. Hoffman, Ph.D.
	Jeffrey R. Stout, Ph.D.
	Maren S. Fragala, Ph.D.
	David H. Fukuda, Ph.D.
	Leonardo P. Oliveira, M.D.
Faculty Supervisor:	Jay R. Hoffman, Ph.D.
Sponsor:	Funding is tentative from MusclePharm Corp.
Investigational Site:	Institute of Exercise Physiology and Wellness
	Sport and Exercise Science
	College of Education and Human Performance
	University of Central Florida
Phone Number(s):	407-823-2367
	201-376-2592 (including after hours)

Introduction:

Researchers at the University of Central Florida (UCF) study many topics. To do this we need the help of people who agree to take part in a research study. You are being asked to take part in a research study which will include about twelve people at UCF. You have been asked to take part in this research study because you are an active young adult male with at least one year of resistance training experience. You must be between the age of 18 and 35 years of age to be included in the research study.

The person doing this research is Adam M. Gonzalez of UCF College of Education and Human Performance. Because the researcher is a doctoral student, he is being guided by Dr. Jay R. Hoffman, a UCF faculty supervisor in the Sport and Exercise Science Department.

What you should know about a research study:
- Someone will explain this research study to you.
- A research study is something you volunteer for.
- Whether or not you take part is up to you.
- You should take part in this study only because you want to.
- You can choose not to take part in the research study.
- You can agree to take part now and later change your mind.
- Whatever you decide it will not be held against you.
- Feel free to ask all the questions you want before you decide.

Purpose of the research study:
The purpose of this study is to examine how the body responds (intramuscular protein signaling, hormonal, and pro-inflammatory immune response) following two different resistance exercise protocols. This study will also examine the effect of a protein/coconut blend ingestion on this response following a resistance exercise protocol. It is unknown whether a hypertrophic protocol (low intensity/high volume) and a strength protocol (high intensity/low volume) differentially stimulate the processes that aid in muscle recovery and remodeling following resistance exercise. Additionally, protein consumption may stimulate these processes and potentially aid in muscle recovery and remodeling. In summary, the investigators of this study are interested in determining how the body responds to two different resistance exercise protocols and if the body responds differently with the addition of a protein/coconut blend ingestion.

What you will be asked to do in the study:
Screening Visit:

You will be asked to read and sign this consent form before any study-related procedures are performed. During the screening visit, the following will be done:

- Physical activity readiness questionnaire (PAR-Q)
- Your age, race and gender will be collected
- Self-reported confidential medical and activity history questionnaire

Upon being admitted to the study, you will be assigned a subject number. You will complete each of the following experimental trials in a random order on three separate occasions:

1. Acute low volume, high intensity resistance exercise protocol + Placebo
2. Acute high volume, low intensity resistance exercise protocol + Placebo
3. Acute high volume, low intensity resistance exercise protocol + Protein/coconut supplement

Study Protocol

All procedures are being done solely for research purposes. You will report to the Human Performance Laboratory (HPL) on four separate occasions. On the first visit, anthropometric assessments including height, body mass, and body fat percentage will be measured. Body fat percentage will be assessed via skinfold analysis using a 4-site skinfold test. You will then be tested for maximal strength (1 repetition maximum: 1-RM) on all lifts involved in the exercise protocol. Prior to maximal strength testing, you will perform a standardized warm-up consisting of 5 minutes on a cycle ergometer against a light resistance, 10 body weight squats, 10 body weight walking lunges, 10 dynamic walking hamstring stretches, 10 body-weight push-ups, and 10 dynamic walking quadriceps stretches.

You will then report to the HPL for three experimental trials on three separate occasions. Prior to each trial, you will be instructed to refrain from all forms of moderate to vigorous exercise for a minimum of 72 hours and to report to the HPL following a 10 hour overnight fast. Experimental trials will be performed in a randomized order. Upon arrival to the HPL for each experimental trial, you will provide a urine sample to be analyzed for hydration status.

During each experimental trial, you will perform the same standardized warm-up followed by a lower-body resistance training protocol designed to incorporating large amounts of muscle mass. Table 1 depicts the training routine for the high intensity (HI) and the high volume (HV) protocols. The HI protocol will utilize a load of 90% 1-RM for sets of 3-5 repetitions with a 3-min rest period length between sets and exercises. The HV protocol will utilize a load of 70% 1-RM for sets of 10-12 repetitions with a 1-min rest period length between sets and exercises. Both protocols will include 6 sets of barbell back squats and 4 sets of bilateral leg press, bilateral hamstring curls, bilateral leg extensions, and seated calf raises. During each trial, if you are unable to complete the desired number of repetitions, spotters will provide assistance until you complete the remaining repetitions. Then, the loads will be adjusted so that you can perform the specific number of repetitions for each set.

Table 1. Resistance Exercise Protocols

Exercise Order	High Intensity Protocol (HI)			High Volume Protocol (HV)		
	Sets x Repetitions	Intensity	Rest Time	Sets x Repetitions	Intensity	Rest Time
1. Barbell Back Squats	6 x 3-5			6 x 10-12		
2. Bilateral Leg Press	4 x 3-5			4 x 10-12		
3. Bilateral Hamstring Curls	4 x 3-5	90% 1-RM	3 minutes	4 x 10-12	70% 1-RM	1 minute
4. Bilateral Leg Extensions	4 x 3-5			4 x 10-12		
5. Seated Calf Raises	4 x 3-5			4 x 10-12		

Following each exercise protocols, you will remain in the HPL for all post-exercise assessments. Each experimental trial will be separated by a minimum of seven days to ensure proper recovery.

Blood samples will be obtained at six time points over the course of the study: baseline (BL), immediately post-exercise (IP), 30 minutes post-exercise (30min), 1 hour post-exercise (1H), 2 hours post-exercise (2H), and 5 hours post-exercise (5H). Fine needle muscle biopsies will be completed at BL, 1H, and 5H. All blood samples and biopsies will be taken at the same time of day to avoid diurnal variations.

You will be provided water and permitted to drink water during experimental trials. You will also be provided a standardized low protein, low carbohydrate breakfast following BL assessments. Post-workout drinks will be provided following IP assessments. Following each protocol, you will be provided either a placebo or a protein/coconut supplement. The supplement is an over-the-counter fluid beverage containing protein and coconut water. The supplement contains 120 calories (6 grams carbohydrates; 20 grams protein; 1 gram fat). The placebo will be identical in taste and appearance, but will contain only coconut flavored water. The supplement and placebo will be made by MusclePharm Corp. The experimental trials will be randomized and the researchers will be blinded regarding which drink is provided. The placebo and supplement drinks will be kept in the refrigerator prior to administration. The use of the supplement in this study is not intended to make or support any health claims.

Electromyography

Electromyography (EMG) is a technique for recording the electrical activity produced by the muscles. EMG activity of the vastus lateralis of the dominant leg will be assessed during maximal strength testing and experimental trials for barbell back squats, bilateral leg press, and bilateral leg extensions. A surface electrode arrangement will be placed on the lower thigh of your dominant leg. The skin beneath the electrodes will be shaved and cleaned with alcohol to maintain a proper signal. EMG assessment does not cause any discomfort and will not interfere with your ability to perform the exercise routine.

Subjective Measures of Soreness, Pain, and Recovery

You will be instructed to assess your subjective feelings of leg soreness and leg pain, as well as your perceived recovery status, using scale questionnaires at BL, IP, 30min, 1H, 2H, and 5H.

Blood Measurements

During each experimental trial, all blood samples will be obtained using a Teflon cannula by personnel trained in phlebotomy with extensive experience in both research and clinical settings. A cannula is a hollow tube, which can be inserted into the opening of a vein and serve as a channel for the transport of fluid. The cannula prevents the need for multiple needle pricks from being performed. The risks associated with the placement of the cannula are not any different than that experienced by a normal blood draw using a needle and syringe. The cannula will be kept open following each blood draw with an infusion of a saline solution. This solution contains salt that is similar to the osmolarity of the blood and acts to minimize potential blood clotting within the cannula that may occur with prolonged use. The cannula placement will not interfere with your ability to perform the exercise routine. BL blood samples will be obtained following a 15-min equilibration period. IP blood samples will be taken within 1 minute of exercise cessation. Following the resistance exercise protocol, you will remain in the HPL for subsequent blood draws. You will be instructed to lie in a supine position for the 15 minutes prior to 30min, 1H, 2H, and 5H blood draws. The total volume of blood that will be obtained during each trial will not exceed 156 ml, and 468 ml for the study as a whole. This is approximately 10.5 tablespoons per trial and 31.6 tablespoons for the entire study. To put the total volume of blood being drawn in proper perspective, one pint (475 ml; 32.1 tablespoons) of blood is typically drawn when donating blood. All blood draws will be conducted under sterile conditions. As an additional

safeguard in preventing contamination, new disposable gloves will be used for all blood draws. All blood samples collected will be frozen until analysis. The discomforts associated with the blood drawing procedures are minimal, but sometimes bruising and infection may occur, and the arm may become sore. This soreness usually resolves in a few days. If it persists, contact your doctor.

Biopsy Procedures

All muscle biopsies will be performed by a trained licensed physician. The fine needle muscle biopsy involves the removal of a small piece of muscle tissue from the vastus lateralis of the dominant leg using a sterile hollow needle. Fine needle biopsies are a technique which allows a researcher to obtain multiple muscle samples without large incisions that accompany other methods. Additionally, the pain level of a fine needle biopsy has been reported to be minimal with individuals comparing it to a "pushing sensation" with most participants engaging in normal physical activity the same day. The area over the outside of the lower thigh muscle (vastus lateralis muscle) will be carefully cleaned. A small amount of lidocaine (anesthetic) will be injected into and under the skin. You will likely experience a small pinching sensation while the numbing agent is injected. After the area is completely numbed, a small incision to the skin will be made and a hollow needle will be placed perpendicular to the muscle until the muscle is pierced. Following placement of the biopsy needle into the biopsy device, the unit will be inserted through the cannula. A muscle sample is obtained by the activation of a trigger button, which unloads the spring and activates the needle to collect a small muscle sample. The biopsy needle is then slid out of the insertion cannula while the cannula is maintained in place, thus avoiding repeated skin punctures. During the time that the sample is being taken (about 5 seconds), you may feel the sensation of pressure in the thigh and on some occasions this is moderately painful. However, the discomfort very quickly passes and you will be quite capable of performing exercise and daily activities. The whole biopsy procedure will be repeated up to 6 times in order to obtain sufficient muscle tissue. There may be some minimal bleeding when the needle is removed which may require application of pressure for a few minutes. Following the biopsy, the incision will be treated with a sterile dressing and wrapped in a bandage.

Dietary Logs

You will be instructed to maintain your normal dietary intake leading up to experiment trials. Then, you will be instructed to record as accurately as possible everything you consume during the 24 hours prior to the first experimental trial. You will be instructed not to eat or drink (except water) within 10 hours of reporting to the HPL for experimental trials. You will be required to duplicate the content, quantity, and timing of your daily diet during the 24 hours prior to the remaining experimental trials.

Location:

All testing will be conducted in the Institute of Exercise Physiology and Wellness at the University of Central Florida.

Time required:

We expect that you will be in this research study for approximately 4 weeks. This will consist of a preliminary trial and three experimental trials separated by at least 7 days. The preliminary trial will last approximately an hour and a half and the three experimental trials may last up to 9 hours.

Funding for this study:

This research study is tentatively being paid for by MusclePharm Corporation.

Risks:

The workout consists of exercises that are common to the training program of individuals with experience in weight lifting. It is expected that you will experience the normal soreness that often accompanies these workouts.

The risks associated with the blood draw may include some momentary pain at the time the needle is inserted into the vein. It is also possible for a bruise to develop at the site that the needle entered the skin or for individuals to report dizziness and possibly faint after the blood is drawn. It is also rare, but possible, to develop minor infections and pain after the blood draw. To minimize the risks, the skin area where the needle or cannula is inserted will be cleaned and prepared with a disinfectant wipe before the needle or cannula is inserted. Needles and cannulas are sterile, and gloves are worn by the person trained in obtaining blood. During experimental trials, a cannula will be used. This is to minimize the number of needle sticks. Upon the removal of the cannula, the puncture site will be covered with a bandage. The total amount of blood drawn during each testing point will not exceed 1.76 tablespoons. The total amount of blood that will be obtained during the study will not exceed 31.6 tablespoons. To put the volume of blood being drawn in proper perspective, one pint (32.1 tablespoons) of blood is typically drawn when donating blood. To reduce the risk of dizziness and fainting from the blood draws, the cannula will be inserted and all blood draws will occur while you are lying flat on your back.

The risks associated with muscle biopsies include momentary pain at the site of sampling during the time the needle is inserted, the possible appearance of a scar and potential bruising and/or soreness, and involuntary muscle contraction at the site of sampling. To limit these risks, a trained physician and technicians will obtain muscle biopsies using sterile techniques. Additionally, the sampling site will be sterilized prior to the procedure. There will be a total of 3-6 samples obtained during each time point for a total of approximately 90 mg (0.09g) of muscle tissue per sample. This will total approximately 270 mg per trial, and approximately 540mg (0.54g) over the course of the study. To put this in perspective, 1 oz is equal to approximately 28g. To reduce the risk of dizziness and fainting, all procedures will occur while you are lying flat on your back. Lidocaine (anesthetic) will be used to minimize the level of discomfort. The risks associated with lidocaine include irritation of the skin, bruising, and, although rare, allergic reaction. Symptoms of an allergic reaction may include a rash, itching, and swelling. Once the local anesthetic wears off, your leg may feel tight and often there is the sensation of a deep bruise or "Charlie Horse". However, the tightness in the muscle usually disappears within 2 days and participants routinely begin exercising at normal capacity within 2 days.

The risks associated with a 10-hour fast are feelings of hunger and tiredness.

The potential risk associated with ingestion of the protein/coconut supplement includes an allergic reaction (itching, rash, cough, diarrhea, etc.). The product does contain coconut, milk, and soy.

The loss of confidentiality is a potential risk of being in the study, but we will do everything to make sure that your information is protected.

Benefits:

There are no direct benefits to participants.

Compensation or payment:

Upon completion of the study, you will receive a $200 payment for your participation. However, if you only complete certain parts of the study, you may be able to be compensated for what you completed. You will receive $25 for completing the first experimental trial, $50 for completing the second experimental trial, and an additional $125 for completing the third experimental trial. No compensation will be provided if you do not complete any experimental trials.

Medical care and compensation for injury

You will be instructed to immediately report any discomforts or adverse effects to the principal investigator. An adverse effect is defined as an intolerable response, perceived to be a direct consequence of participation in this study. If immediate assistance is needed it will be provided via the emergency medical system. For non-emergency injuries, you must seek treatment from your own physician. If you suffer a physical injury as a result of participation in this study, you may be reimbursed for medical expenses to treat the injury, to the extent not paid by your insurance. You should receive medical care in the same way as you would normally. No funds have been set aside for payments or other forms of compensation (such as for lost wages, lost time, or discomfort). You do not give up any of your legal rights by signing this consent form. Adverse events/side effects will be reported to the IRB immediately upon notification.

Alternatives:

There are no other alternatives available outside of this study.

Cost:

There is no cost to you to be in the study.

Confidentiality:

Records of your participation in this study will be held confidential so far as permitted by law. The study investigator or it's designee, and, under certain circumstances, New England Institutional Review Board (NEIRB), will be able to inspect and have access to confidential data that identifies you by name. Any publication or presentation of the data will not identify you. By signing this consent form, you authorize the study investigator to release your medical records to the sponsor and the IRB.

The results of this study will be published as a group as part of a scientific publication. No individual results will be published or shared with any person or party. All information attained from the medical and activity questionnaire or performance tests will be held in strict confidence. Individual results will remain confidential and only be relayed to you upon request. All medical and activity questionnaires, as well as data collection sheets will be kept in a locked cabinet during and following the study. All information will be destroyed 5 years from the end of the study and not used for other research purposes. Participant folders and blood storage tubes will be marked with an I.D. number to protect

against a breach of confidentiality and the ID number will be removed upon disposal. Participant names and I.D. numbers will be stored apart from the blood samples. The identifiers will be removed from the samples and destroyed when the samples are disposed of.

Study contact for questions about the study or to report a problem:
If you have questions, concerns, or complaints, or think the research has hurt you, talk to Adam M. Gonzalez, Doctoral Student, Exercise Physiology, College of Education and Human Performance at (201) 376-2592 or by email at adam.gonzalez@ucf.edu. You may also contact Dr. Jay R. Hoffman, Institute of Exercise Physiology and Wellness, Sport and Exercise Science at (407) 823-2367 or by email at jay.hoffman@ucf.edu.

IRB contact about your rights in the study or to report a complaint:
If you have questions about your rights as a research subject, or other concerns about the research, you can contact the New England Institutional Review Board (NEIRB) at 1-800-232-9570.

Withdrawing from the study:
You have the right to discontinue participation without penalty, regardless of the status of the study. Your participation in the study may also be terminated at any time by the researchers in charge of the project or by New England IRB. This could be based upon your refusal to follow study instructions or follow study protocol.

For Students and Employees of University of Central Florida:
Your participation in this study is voluntary. You are free to withdraw your consent and discontinue participation in this study at any time without prejudice or penalty. Your decision to participate or not participate in this study will in no way affect your continued enrollment, grades, employment, or your relationship with individuals who may have an interest in this study. _____ initials

(Please note you will be participating in this study on your own time; not during regular working hours or class time.)

VOLUNTEER'S STATEMENT:

I have been given a chance to ask questions about this research study. These questions have been answered to my satisfaction. I may contact Adam Gonzalez if I have any more questions about taking part in this study. Adam Gonzalez or the company he is employed by is being paid by the sponsor for my participation in this study.

I understand that my participation in this research project is voluntary. I know that I may quit the study at any time without harming my future medical care or losing any benefits to which I might be entitled. I also understand that the investigator in charge of this study may decide at any time that I should no longer participate in this study.

If I have any questions about my rights as a research subject in this study I may contact:

New England Institutional Review Board
Telephone: 1-800-232-9570

By signing this form, I have not waived any of my legal rights.

I have read and understand the above information. I agree to participate in this study. I understand that I will be given a copy of this signed and dated form for my own records.

_____   _____
Study Participant (signature)   Date

_____
Print Participant's Name

_____   _____
Person who explained this study (signature)   Date

APPENDIX D: MEDICAL HISTORY QUESTIONNAIRE AND PAR-Q

Confidential Medical and Activity History Questionnaire

Participant #_____

When was your last physical examination? _____

1. **List any medications, herbals or supplements you currently take or have taken the last month:**

   Medication   Reason for medication

   _____   _____
   _____   _____
   _____   _____
   _____   _____

2. **Are you allergic to any medications? If yes, please list medications and reaction.**

3. **Please list any allergies, including food allergies that you may have?**

4. **Have you ever been hospitalized? If yes, please explain.**

   Year of hospitalization   Reason

   _____   _____
   _____   _____
   _____   _____

5. **Illnesses and other Health Issues**

   List any chronic (long-term) illnesses that have caused you to seek medical care.

Approved by NEIRB on _8/20/14_
As Is _X_ As Revised _____ Initials _____

1

Human Performance Laboratory
University of Central Florida

Have you ever had (or do you have now) any of the following. Please circle le
questions that you do not know the answer to.

Sickle cell anemia	yes	no
Cystic fibrosis	yes	no
Water retention problems	yes	no
Heart pacemaker	yes	no
Epilepsy	yes	no
Convulsions	yes	no
Dizziness/fainting/unconsciousness	yes	no
Asthma	yes	no
Shortness of breath	yes	no
Chronic respiratory disorder	yes	no
Chronic headaches	yes	no
Chronic cough	yes	no
Chronic sinus problem	yes	no
High blood pressure	yes	no
Heart murmur	yes	no
Heart attack	yes	no
High cholesterol	yes	no
Diabetes mellitus or insipidus	yes	no
Rheumatic fever	yes	no
Emphysema	yes	no
Bronchitis	yes	no
Hepatitis	yes	no
Kidney disease	yes	no
Bladder problems	yes	no
Tuberculosis (positive skin test)	yes	no
Yellow jaundice	yes	no
Auto immune deficiency	yes	no
Anemia	yes	no
Endotoxemia	yes	no
Thyroid problems	yes	no
Hyperprolactinemia	yes	no
Anorexia nervosa	yes	no
Bulimia	yes	no
Stomach/intestinal problems	yes	no
Arthritis	yes	no
Back pain	yes	no
Gout	yes	no
Hepatic encephalopathy	yes	no
Mania	yes	no
Hypermania	yes	no
Monosodium glutamate hypersensitivity	yes	no
Seizure disorders	yes	no

2

Human Performance Laboratory
University of Central Florida

Any others (specify):_____

_____
_____

Do you smoke cigarettes or use any other tobacco products?	yes	no
Do you have a history of drug or alcohol dependency?	yes	no
Do you ever have any pain in your chest?	yes	no
Are you ever bothered by racing of your heart?	yes	no
Do you ever notice abnormal or skipped heartbeats?	yes	no
Do you ever have any arm or jaw discomfort, nausea, Or vomiting associated with cardiac symptoms?	yes	no
Do you ever have difficulty breathing?	yes	no
Do you ever experience shortness of breath?	yes	no
Do you ever become dizzy during exercise?	yes	no
Are you pregnant?	yes	no
Is there a chance that you may be pregnant?	yes	no
Have you ever had any tingling or numbness in your arms or legs?	yes	no
Has a member of your family or close relative died of heart problems or sudden death before the age of 50?	yes	no
Has a health care practitioner ever denied or restricted your participation in sports for any problem	yes	no

If yes, please explain: _____

_____

Are you presently taking any nutritional supplements or ergogenic aids? (if yes, please detail. _____

_____
_____
_____
_____

Approved by NEIRB on 8/21/14
As Is _k_ As Revised _____ Initials _____

PAR-Q & YOU

(A Questionnaire for People Aged 15 to 69)

Regular physical activity is fun and healthy, and increasingly more people are starting to become more active every day. Being more active is very safe for most people. However, some people should check with their doctor before they start becoming much more physically active.

If you are planning to become much more physically active than you are now, start by answering the seven questions in the box below. If you are between the ages of 15 and 69, the PAR-Q will tell you if you should check with your doctor before you start. If you are over 69 years of age, and you are not used to being very active, check with your doctor.

Common sense is your best guide when you answer these questions. Please read the questions carefully and answer each one honestly: check YES or NO.

YES	NO		
☐	☐	1.	**Has your doctor ever said that you have a heart condition _and_ that you should only do physical activity recommended by a doctor?**
☐	☐	2.	**Do you feel pain in your chest when you do physical activity?**
☐	☐	3.	**In the past month, have you had chest pain when you were not doing physical activity?**
☐	☐	4.	**Do you lose your balance because of dizziness or do you ever lose consciousness?**
☐	☐	5.	**Do you have a bone or joint problem (for example, back, knee or hip) that could be made worse by a change in your physical activity?**
☐	☐	6.	**Is your doctor currently prescribing drugs (for example, water pills) for your blood pressure or heart condition?**
☐	☐	7.	**Do you know of _any other reason_ why you should not do physical activity?**

If you answered

YES to one or more questions

Talk with your doctor by phone or in person BEFORE you start becoming much more physically active or BEFORE you have a fitness appraisal. Tell your doctor about the PAR-Q and which questions you answered YES.

- You may be able to do any activity you want — as long as you start slowly and build up gradually. Or, you may need to restrict your activities to those which are safe for you. Talk with your doctor about the kinds of activities you wish to participate in and follow his/her advice.
- Find out which community programs are safe and helpful for you.

NO to all questions

If you answered NO honestly to all PAR-Q questions, you can be reasonably sure that you can:
- start becoming much more physically active — begin slowly and build up gradually. This is the safest and easiest way to go.
- take part in a fitness appraisal — this is an excellent way to determine your basic fitness so that you can plan the best way for you to live actively. It is also highly recommended that you have your blood pressure evaluated. If your reading is over 144/94, talk with your doctor before you start becoming much more physically active.

DELAY BECOMING MUCH MORE ACTIVE:
- if you are not feeling well because of a temporary illness such as a cold or a fever — wait until you feel better; or
- if you are or may be pregnant — talk to your doctor before you start becoming more active.

PLEASE NOTE: If your health changes so that you then answer YES to any of the above questions, tell your fitness or health professional. Ask whether you should change your physical activity plan.

Informed Use of the PAR-Q: The Canadian Society for Exercise Physiology, Health Canada, and their agents assume no liability for persons who undertake physical activity, and if in doubt after completing this questionnaire, consult your doctor prior to physical activity.

No changes permitted. You are encouraged to photocopy the PAR-Q but only if you use the entire form.

NOTE: If the PAR-Q is being given to a person before he or she participates in a physical activity program or a fitness appraisal, this section may be used for legal or administrative purposes.

"I have read, understood and completed this questionnaire. Any questions I had were answered to my full satisfaction."

NAME _____

SIGNATURE _____ DATE_____

SIGNATURE OF PARENT _____ WITNESS _____
or GUARDIAN (for participants under the age of majority)

> **Note: This physical activity clearance is valid for a maximum of 12 months from the date it is completed and becomes invalid if your condition changes so that you would answer YES to any of the seven questions.**

Approved by NEIRB on ___8/20/14___
As Is _X___ As Revised _____ Initials ____

APPENDIX E: COPYRIGHT LICENSE AGREEMENTS

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Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
High-res required	no
Figures	Figure 1. Development and Function of Monocyte Subsets in Mice
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