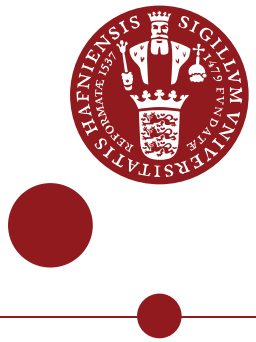
UNIVERSITY OF COPENHAGEN
FACULTY OF SCIENCE



Master's thesis:

The ageing muscle: Macrophage kinetics and local inflammation in response to resistance training

Den aldrende muskel: Makrofager og lokal inflammation i forbindelse med styrketræning.

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Preface

This study was performed at Institute of Sports Medicine Copenhagen, Bispebjerg Hospital.

This thesis could not have been written without the help from a number of people. I want to thank everyone at the institute for all help and guidance received during my time here. A special thanks to my supervisor at the hospital: Michael Kjær, my supervisor at Copenhagen University: Jørgen Wojtaszewski, everyone involved in the LISA study and everyone from the ISMC "muscle group".

Abstract:

Macrophages play a central role in skeletal muscle repair. Macrophages secrete pro- and anti-inflammatory cytokines important for successful regeneration of muscle fibers. Age related muscle loss has been associated with increased systemic levels of pro-inflammatory cytokines similar to those expressed by macrophages. It is speculated that an increased inflammatory environment in local tissue impairs muscle regeneration in elderly, contributing to reductions in muscle mass. Resistance training is believed to enhance the anti-inflammatory properties of the muscle, protecting against or counteracting an inflammatory environment.

Purpose: The primary aim of this study was to investigate local macrophage content following an acute bout of unaccustomed exercise and after a 12 week training intervention in young and elderly subjects. Secondarily, we wanted to develop a method for detection of macrophage subtypes in human muscle cross sections.

Methods: Data from two separate studies were analyzed. Study 1: *Acute study*. 27 elderly men (mean ± SD: age = 70.3 ± 6.6) performed one bout of unilateral leg extension consisting of 5 x 12 concentric repetitions (70% 1RM) followed by 4 x 6 eccentric repetitions (110% 1RM). 4 biopsies from the vastus lateralis were analyzed: one sample prior (PRE) to the training bout and 3 samples in the days following the training bout (1day, 4days and 7days). *Study 2*: *training study*. 10 young (mean ± SD: age = 22.4 ± 1.8) and 10 elderly men (mean ± SD: age = 66.6 ± 4.2) were subjected to 36 training sessions (12 weeks with 3 sessions/week). Biopsies were taken before the training intervention (PRE) and following the training intervention (POST). For both studies, immunohistochemistry was performed for total macrophages (CD68+), anti-inflammatory macrophages (CD68+CD163+) and pro-inflammatory macrophages (CD68+CD163-). Additionally, iNOS and TNF-α was tested as possible markers for pro-inflammatory macrophages.

Results: *Acute study:* Infiltration of CD68+ cells increased significantly within 24 hours (0.037 vs 0.055 cells/fiber, P = 0.046). Gradual increases were observed during the following days, with highest detected counts 7 days following the training bout (0.104 cells/fiber, P < 0.001). CD68+CD163+ cells were significantly elevated on day 4 (0.047 cells/fiber) and day 7 (0.069 cells/fiber) when compared to PRE (0.020 cells/fiber) and day 1 (0.029 cells/fiber) . CD68+CD163- baseline results (0.014 cells/fiber) cells tended to increase on day 4 (0.022, P = 0.051) and significantly increased on day 7 (0.026 cells/fiber, P = 0.014). *Training study:* Local changes in CD68+ cells were observed for the OLD group (0.047 cells/fiber vs 0.068 cells/fiber, P = 0.042) but not for the young group post exercise. No changes were observed over time for CD68+CD163+ cells in any of the groups, but in general OLD muscle contained more CD68+CD163+ cells compared to young muscle (P = 0.020). At baseline, young muscle contained more CD68+CD163- cells than muscle in elderly (0.019 cells/fiber vs 0.009 cells/fiber, P = 0.002).

Conclusion: These findings illustrate an increased local macrophage content following one physiological bout of resistance training. In contrast to our expectations, there was generally no sign of local accumulated macrophage content following a 12 week training intervention. Differences in pro- and anti-inflammatory macrophage content when comparing young and elderly may play a role for changes in muscle structure/quality as seen with progressing age. Finally, no successful staining protocol was evident using iNOS or TNF-α for pro-inflammatory macrophages.

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1. Introduction

The number of people aged ≥ 60 years is expected to grow rapidly worldwide. It is estimated that within the year 2050, people older than 60 years will triple compared to now, with people aged +85 being the most rapidly expanding demographic group altogether (Garatachea et al. 2015). Combined, with an increased life expectancy (WHO 2013) these demographic shifts present new challenges to public institutions, health care institutions and society in general. Healthy aging is essential to maintain independent daily function and life quality as we age. From an economic standpoint healthy aging may prolong work force activity and benefit medical and health expenses for public institutions. Therefore, investigating the mechanisms responsible for progressed muscle loss and loss of physical function in relation to age are of great relevance to counteract or postpone symptoms.

Although numerous studies have investigated the aging muscle, the exact mechanisms causing an age related muscle loss have not yet been clarified. Several factors such as anabolic resistance, muscle denervation, diminished satellite cells and increased pro-inflammatory cytokines have all been proposed as contributing factors (Garatachea et al. 2015). Although the main focus of this assignment will be on macrophages and inflammation, other topics will be presented. This is necessary to understand and investigate the complex overlapping physiological mechanisms believed to contribute to age related muscle loss also referred to as *Sarcopenia.*

1.1 What is Sarcopenia

All body movements are produced by contractions of skeletal muscle. The amount of skeletal muscle mass is considered to be a major determinant of muscle strength which is important to carry out everyday tasks and maintain high quality of life. It is well known that human skeletal muscle mass starts to decrease at minimal speed from age 25-30, accelerating as we come closer to the age of 60 (Lexell, Taylor, and Sjöström 1988; Garatachea et al. 2015). A study by Dechenes (Deschenes 2004) specifies these findings. They have found that muscle strength is well maintained until we reach an age of 50, however, over 10 years (between our 50th and 60th year) an increased decline in strength is detected. This decline is even more pronounced beyond the age of 60 years

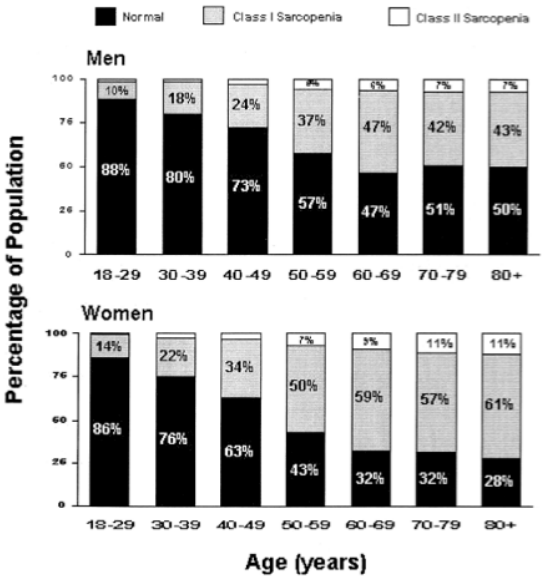

Figure 1. Prevalence of sarcopenia for men (top) and women (bottom) at different age groups. Class I sarcopenia: muscle mass between -one to -two SD compared to young adult mean. Class II sarcopenia: muscle mass below two SD compared to young adult mean. Illustration from Janssen et al. (2002).

(Deschenes 2004). Also, a study by Grimby et al. has shown that by the age of 60 total muscle mass has decreases by 25-30% before we reach an age of 70.

Sarcopenia is a term created to refer to age related loss of muscle mass, quality and strength (Garatachea et al. 2015). Although Sarcopenia has not been internationally accepted as a definition in medical literature it is still used in clinical settings defined as the combined symptoms of impaired walking capability (low gait speed or endurance in a 6-min walk) and a muscle mass lower than 2 standard deviations from the mean measured in young men between age 20-30 years (Baumgartner et al. 1998; Garatachea et al. 2015). An epidemiologic study by Baumgartner tried to clarify the extent of Sarcopenia in the general population. The study shows that prevalence of Sarcopenia was seen in 25% of people younger than 70 years and more than 50% of people aged 80 years and older (Baumgartner et al. 1998). These numbers are supported by a study by Janssen in which almost 15.000 subjects aged 18 and older were examined (Janssen, Heymsfield, and Ross 2002). Results are shown in figure 1 and illustrate the prevalence of Sarcopenia in the general population at different ages. The figure not only emphasizes that Sarcopenia poses a threat to a large amount of elderly, but also illustrates the accelerated development of Sarcopenia until we reach a certain age.

Any impairment in the functional properties of skeletal muscle results in some degree of immobility. Accidents and injuries occurring to Sarcopenic elderly has been linked to an increased loss of independent daily function. This affects life quality and increases hospitalization, morbidity and even mortality (Deschenes 2004; Janssen, Heymsfield, and Ross 2002). Sarcopenia therefore poses a major public health problem that will have to be addressed to increase life quality and independency for the individual but also for public health care institutions.

The mechanisms causing sarcopenia has been investigated in a large number of studies. The following will describe the observed changes in fiber size and count as an effect of ageing. This will be accompanied by a number of studies investigating the possible mechanisms causing age related changes in muscle. One of the components making Sarcopenia difficult to investigate is its long term duration and gradually increasing influence with age. These properties make it hard to identify the exact mechanisms initiating, maintaining and enhancing the physiological changes during ageing. Many studies have addressed sarcopenia by isolating a certain topic or pathway. Others have tried to analyze the interaction of many physiological mechanisms and pathways (Garatachea et al. 2015). Both may be necessary to get a better understanding of the complex condition seen in the ageing muscle. It is important to note that several studies included in the following have investigated causes for Sarcopenia in extreme research protocols. Such protocols include cell necrosis, partial muscle excision, satellite cell depletion etc. Many of such studies have been carried out in animals, questioning their applicability to humans. In addition to that strength training protocols carried out in humans often reflects the extremes of the spectrum with limited parallels to muscle damage emerging in

everyday life. Such protocols may be necessary to examine basic principles, all adding to our current knowledge of Sarcopenia.

1.2 Human muscle structure and physiology

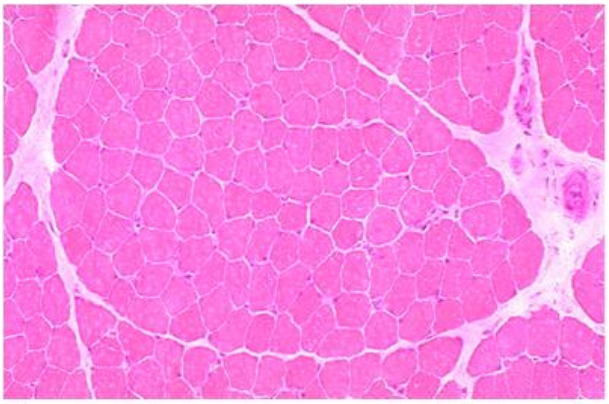

Figure 2. Skeletal muscle cross section. Skeletal muscle consists of many fascicles. A muscle fascicle is formed by a number of myofibers joined together by endomysium (connective tissue). The fascicle is surrounded by an even larger layer of connective tissue (perimysium) connecting the fascicle with other neighboring fascicles.

One of the current available techniques to analyze human skeletal muscle is the study of muscle cross sections. To study muscle cross sections in a microscopic scale, tissue samples have to be collected, obtained through invasive muscle biopsies. An example of a cross section is displayed in figure 2. The human skeletal muscles are composed of numerous cells called myofibers. In a cross section with aligned fibers most of these will have a pentagon angular shape (see fig. 2). Each myofiber is coated by a cell membrane called sarcolemma which is surrounded by endomysium. The endomysium is a type of connective tissue that joins together the individual myofibers into fascicles as the one illustrated in figure 2. A part of the endomysium is referred to as the basal lamina. This layer and the sarcolemma surround the myofibers and form a small space between them, referred to as the satellite cell niche. This area will be of importance when we discuss satellite cells. Fascicles are surrounded by another even larger connective tissue layer called the perimysium which connects fascicles with one another. Myofibers contain myosin and actin which are vital to muscle contractions. Muscle contractions occur when action potentials from motor neurons are transmitted to the myofibers through the neuromuscular junction. The myofibers are then depolarized causing the sarcoplasmatic reticulum to release calcium ions which bind to actin filaments initiating the cross bridge cycles resulting in a contraction of the muscle.

Skeletal muscle fibers can be classified either as fast twitch (type II) or slow twitch fibers (type I). Briefly, type II muscle fibers are characterized by a large fiber diameter and a high contractile force and velocity, whereas type I is smaller and produces a lower force and velocity. In young muscle (25 years) type 2 fiber size is usually ranged between 3000 – 5000 um^2 and type 1 fiber size within the range of 3000 – 4000 um^2 (Andersen 2003). For both fiber types sizes however, big variations occur - as illustrated in figure 3 (Porter, Vandervoort, and Lexell 1995).

All muscle cells are innervated by alpha motor neurons whose cells bodies are located in the spinal cord. The motor nerves branch - with each branch innervating a single muscle fiber. The motor nerve and the fibers innervated by it compose a motor unit. In young muscle, fibers innervated by the same motor neuron are randomly distributed and intertwined with fibers from other neurons.

1.3 Observed changes in the ageing muscle

Studies have shown that atrophy is evident in single fibers with age – meaning that the cross sectional area of the muscle have been reported to decrease over time (Lexell, Taylor, and Sjöström 1988; Andersen 2003; Deschenes 2004). Additionally, studies have shown that single fiber atrophy is more predominant in type II fibers than in type I fibers (Andersen 2003; Porter, Vandervoort, and Lexell 1995). Andersen compared muscles fibers from a group of elderly (age 88) with a group of young (age 25) subjects and found that the type II fibers of the elderly were reduced to 43% of the young type II fibers, whereas the type I fibers in the older group were reduced to 75% of the younger group, observing a type I and type II fiber size at 2891 um^2 and 1704 um^2, respectively (Andersen 2003). The fiber sizes found in the study by Andersen are consistent with data from Lexell, provided in figure 3. These results indicate that type II fibres are more exposed to reductions in fiber size than type I. Considering that type II fibers are more dominant in force and power generation, this reduction could contribute to our understanding of the loss of power and strength seen in elderly.

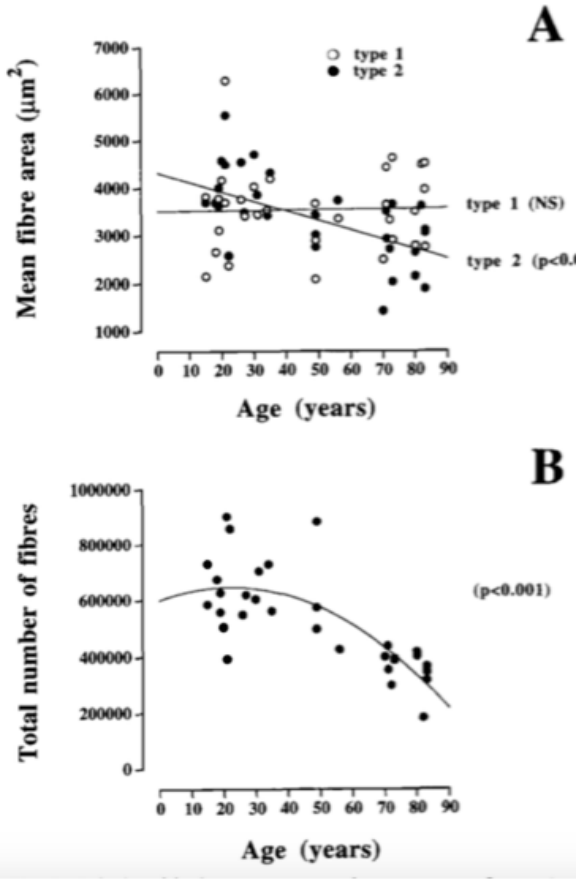

Figure 3. Relationship between (**A**) age and mean area of type 1 and type 2 muscle fibers and (**B**) between age and total numbers of muscle fibers. From Lexell et al. (1988)

Another explanation for ageing muscle atrophy has been a reduction in muscle fiber count (Lexell, Taylor, and Sjöström 1988). The reduction in muscle fibers has been linked to a degeneration of motor neurons in the spinal cord. An early study by Tomlinson and Irving has shown that until the age of 60 there is no evidence of a diminishing motor neuron population, however when above 60 years of age, several cases have shown a 50% decrease of motor neurons compared to the counts conducted in adult life or middle age (Tomlinson and Irving 1977). This reduction in functioning motor units is considered to be a possible explanation for the reduction of muscle fibers in the ageing muscle, as denervated muscle cells ultimately disappear. According to Porter (Porter, Vandervoort, and Lexell 1995) the loss of muscle in elderly is mainly determined by the number of fibers and to a lesser extent by the number/size of type II fibers, however, they both seem to contribute to decreases observed in human muscle cross sectional area.

1.3 Possible mechanisms underlying changes in muscle mass and quality

1.3.1 Satellite cells

The human skeletal muscle fiber is one of few cells types that are multinucleated. Myonuclei are located in the periphery of the fiber in the space between the myofibrils and sarcolemma. Muscle hypertrophy and repair has been associated with, and is dependent on, addition of newly formed myonuclei (Shenkman et al. 2010). Incorporation of new myonuclei is provided by myogenic cells derived from muscle stem cells also known as satellite cells. Satellite cells have been investigated extensively because of their important role in muscle regeneration and hypertrophy; however their potential role in the ageing muscle has not yet been clarified.

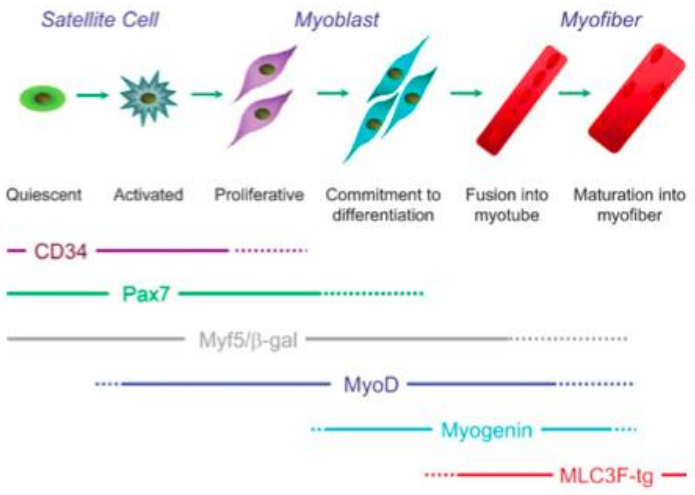

Figure 4. Schematic of satellite cell fate and markers typical of each state. Quiescent satellite cells are located in the satellite cell niche - between the sarcolemma and the basal lamina.

Satellite cells have the ability to proliferate, differentiate and fuse into myotubes leading to new myofiber formation or reconstruction of existing damaged myofibers (Yin, Price, and Rudnicki 2013; Verdijk et al. 2014). In relation to satellite cells it is important to distinguish between muscle regeneration and muscle remodeling in response to strength training. Muscle regeneration is induced by damage leading to myonecrosis of the muscle fibers. Such damage is difficult to provoke under physiological circumstances (Grounds 2014). Muscle loading with no myonecrosis is sometimes referred to as muscle remodeling/repair, characterized by no regenerating myofibers. Muscle regeneration is often misused covering situations of minor muscle tissue damage or situations with small increases in muscle mass. This is incorrect according to Grounds (Grounds 2014) defining muscle regeneration only as a response to situations where myonecrosis has first occured. The importance of satellite cells in regeneration of muscle tissue is emphasized in studies where ablation of the total satellite pool completely inhibits muscle regeneration (Sambasivan et al. 2011) however this has only been investigated in animal studies.

In healthy, unstressed muscle, satellite cells are in a quiescent, inactive state located around the myofibers between the sarcolemma and basal lamina. This is also referred to as the stem cell niche (Yin, Price, and Rudnicki 2013; Conboy 2008). Upon activation, satellite cells change into satellite cell derived myoblasts also referred to as myogenic precursor cells (MPCs) (Saclier et al. 2013). Activation of satellite cells is initiated by muscle trauma, stretch or overload. When damage is minimal, satellite cells and their progeny

fuse with existing myofibers. Such injuries occur regularly during normal muscle activity, suggesting a continuous demand of satellite cells for ongoing repair throughout life. On the other side, massive damage to muscle fibers will stimulate myogenic precursor cells to fuse with each other to form new myofibers (Shefer et al. 2006; Grounds 2014). This is relevant in situations where muscle damage has induced cell necrosis.

Myogenic precursor cells can self-renew and go back to quiescence which is important to replenish the satellite pool. They can also commit to differentiation to become functional in myotube and myofiber formation (Yin, Price, and Rudnicki 2013; Zammit, Partridge, and Yablonka-Reuveni 2006). Collins and colleagues have shown that as few as seven satellite cells transplanted into a mouse can generate over a 100 new myofibers containing thousands of myonuclei thereby illustrating the proliferative properties of the satellite cell (Collins et al. 2005). Myofiber formation is believed to happen in two stages. First, individual differentiated myoblasts fuse with one another to create myotubes with few nuclei. Secondly, additional myoblasts are incorporated into the myotube forming a mature myofiber with contractile proteins (Yin, Price, and Rudnicki 2013; Zammit, Partridge, and Yablonka-Reuveni 2006). However, myotubes can also fuse with existing myofibers repairing the damaged fibers (Shefer et al. 2006). As previously stated, it is widely accepted that satellite cells play an important role in muscle regeneration and that muscle regeneration in the ageing muscle is impaired, however less is known about the mechanisms responsible for the impairment (Wagers and Conboy 2005; Conboy 2008).

The expression of different transcription factors allows for investigation of satellite cells and their contribution to tissue regeneration. In general, satellite cells can be identified from their unique position in the skeletal muscle tissue, located in the stem cell niche. As illustrated in figure 4, quiescent satellite cells, activated satellite cells and proliferating myogenic precursor cells all express transcription factor Pax7 (Zammit, Partridge, and Yablonka-Reuveni 2006). In some studies, satellite cell activation and proliferation is referred to as the early regenerative phase (Saclier et al. 2013). When satellite cells are activated, they rapidly initiate the expression of transcription factor MyoD. Compared to the Pax7 expression this can be useful to distinguish quiescent satellite cells from activated cells. Finally, myogenin marks the onset of MPC differentiation and fusion (Zammit, Partridge, and Yablonka-Reuveni 2006). The stage in which MPCs express myogenin is sometimes referred to as the late regenerative phase.

Verdijk et al. have investigated the human muscle satellite cell content over the entire lifespan. According to this study satellite cell content in type II muscle fibers decreases with age, whereas type I satellite cell content remains unchanged. Verdijk's study also investigates fiber type sizes and finds a decrease in type II fibers. They conclude that there might be a relation between the reduction in fiber type II size and the reduced satellite cell content (Verdijk et al. 2014). Considering that sarcopenia is age related muscle loss and the theory that satellite cells are responsible for muscle growth and maintenance throughout life, it is

reasonable to hypothesize that loss of satellite cells is a major determinant for sarcopenia. However, several studies have shown no sign of age-related loss of satellite cells (Roth et al. 2001; Wagers and Conboy 2005). Also, a recent study in mice has shown that if satellite cells are depleted at an early age and these mice age naturally no sign of either accelerated Sarcopenia or decrease in muscle size is seen; however the regenerative capacity of muscles is impaired (Fry et al. 2015). This contradicts the previous beliefs stating that sarcopenia is related to a loss of satellite cells. Instead recent focus has been aimed at factors affecting the satellite cell niche environment and the myonuclear domain (van der Meer, Jaspers, and Degens 2011) rather than the amount of satellite cells present in the myofibers. With a location within the niche the satellite cell is surrounded by other cells and the extracellular matrix which provide biochemical and biophysical signals that direct regeneration and self-renewal (Parker 2015). Therefore several other factors such as notch signaling (Parker 2015; Hikida 2011; Wagers and Conboy 2005), transforming growth factor beta (Burks et al. 2011), insulin-like growth factor 1 (Burks et al. 2011) and macrophages (Saclier et al. 2013; Chazaud 2014) have received increased attention as possible mechanisms regulating satellite proliferation, differentiation and fusion.

1.3.2 Inflammation

Inflammation is the body's non-specific response to a wide variety of tissue damage produced by mechanical, chemical or microbial stimuli. It is characterized by the movement of immune cells and fluids from blood into the site of damaged tissue, causing pain, redness, swelling and reduced function (Ferrero-Miliani et al. 2007). Local inflammation is believed to be an initial physiological protective response to tissue damage; however local inflammatory factors also mediate the repair of the damaged tissue (Mosser and Edwards 2008). In order to carry out these functions the process of inflammation needs to be strictly controlled. An insufficient inflammatory response to damage could lead to progressive tissue destruction by harmful stimuli, whereas an overly active response could become self-destructive and fatal to the host.

The strictly orchestrated inflammatory response to damage is caused by cells from the immune system. Immune system cells release small proteins known as cytokines, these are crucial for cell communication and regulation of inflammation. Cytokines usually act in a paracrine and/or autocrine manner modulating the behavior of local neighboring cells but also in the regulation of monocytes into macrophages. The list of discovered cytokines is long, Mosser (Mosser and Edwards 2008) and Mantovani (Mantovani et al. 2004) have provided a brief overview of some of the cytokines known to play important roles for macrophage activation. Cytokines are often classified as being either pro-inflammatory (e.g. TNF-α, Interleukin (IL)-1,) or anti-inflammatory (e.g. IL-10, IL-4, IL-1ra) depending on their role in the response to damage, however some cytokines have been suggested to have a pro- and anti-inflammatory role (such as lL-6 (Munoz-

Canoves et al. 2013)) illustrating the complex nature of different cytokines. Studies have shown that local inflammation is accompanied by a systemic response also referred to as the acute phase response. This response activates the production of other proteins that can be detected in the systemic circulation (Petersen and Pedersen 2005). The acute phase response demonstrates that cytokines do not only carry out their function in local tissue, they also promote changes in the circulation, regulating a large number of physiological pathways to adapt to exercise or injury.

C-reactive protein (CRP) is a non-specific acute-phase response to most forms of inflammation, infection and tissue damage. It is produced by hepatocytes in the liver in response to pro-inflammatory cytokines - predominantly IL-6 (Pepys and Hirschfield 2003). A study including 468 healthy young adults found CRP median concentrations at 0.8 mg/l. In this study, 90% of samples contained less than 3 mg/l and 99% contained less than 10 mg/l (Shine, de Beer, and Pepys 1981). Following an acute phase stimulus, values may increase with up to 500 mg/l depending on the type of bacteria or infection causing the inflammation (Pepys and Hirschfield 2003; Clyne and Olshaker 1999). CRP levels increase rapidly and peak around 48 hours after infection and when the stimulus for CRP production ceases CRP concentration falls rapidly. In most diseases, the circulating value of CRP (although unspecific) reflects ongoing inflammation or tissue damage more accurately than other laboratory parameters, making it very useful marker for detection of inflammation and/or disease screening (Pepys and Hirschfield 2003). Increased basal levels of pro-inflammatory cytokines and CRP has been associated with reduced strength and muscle fatigue in elderly illustrating the possible link between systemic inflammation and sarcopenia (Bautmans et al. 2005; Visser et al. 2002).

1.3.2.1 Inflammation scenarios

Numerous repeated muscle contractions as seen in aerobic and anaerobic exercise change the systemic cytokine profile (Petersen and Pedersen 2005). Figure 5a, illustrates the changes in systemic cytokine profile in the hours following endurance exercise. According to Petersen (Petersen and Pedersen 2005), IL-6 is the first cytokine present in circulation. The level of circulating IL-6 increases exponentially (up to 100-fold) depending on duration and intensity of the performance. The increase of IL-6 is followed by smaller increases in anti-inflammatory cytokines IL-10 and IL-1ra. Post exercise increases in cytokines return to baseline levels within a few hours. As previously stated, IL-6 has both pro- and anti-inflammatory properties. During exercise, IL-6 has been suggested to originate from the contracting skeletal muscle tissue, classifying IL-6 as a myokine (Petersen and Pedersen 2005). Although still debated, the IL-6 myokine has been suggested to exert inhibitory effects on TNF-α and IL-1 production. Additionally, IL-6 has been associated with production of other anti-inflammatory cytokines such as IL-1ra and IL-10 shown in figure 5

(Petersen and Pedersen 2005). These effects however, have been linked only to short termed peaks of IL-6 (Munoz-Canoves et al. 2013). The complexity of IL-6 is evident in studies where high doses or long term exposure to IL-6 is related to muscle proteolysis, revealing the complications of the IL-6 response and its functional properties (Munoz-Canoves et al. 2013).

Systemic (chronic) low grade inflammation, illustrated in figure 5b, has been introduced as a term for conditions in which a typically two- to threefold increase in the systemic concentrations of pro-inflammatory cytokines such as TNF-α, IL-1, IL-6 and CRP is reflected. Chronic low grade inflammation has been linked to various metabolic diseases and muscle loss (Petersen and Pedersen 2005). Little is known about the stimuli responsible for increased levels of pro-inflammatory cytokines, but it is assumed that adipose tissue is the origin of pro-inflammatory TNF-α, contributing to increased inflammation levels (Petersen and Pedersen 2005). Chronic levels of inflammation have especially been associated with ageing in elderly (Degens 2010; Garatachea et al. 2015). In relation to that the term "inflammaging" has been used to describe chronic upregulation of the inflammatory response observed with advancing age. The mechanisms responsible for "inflammaging" however remains to be elucidated (Minciullo et al. 2016).

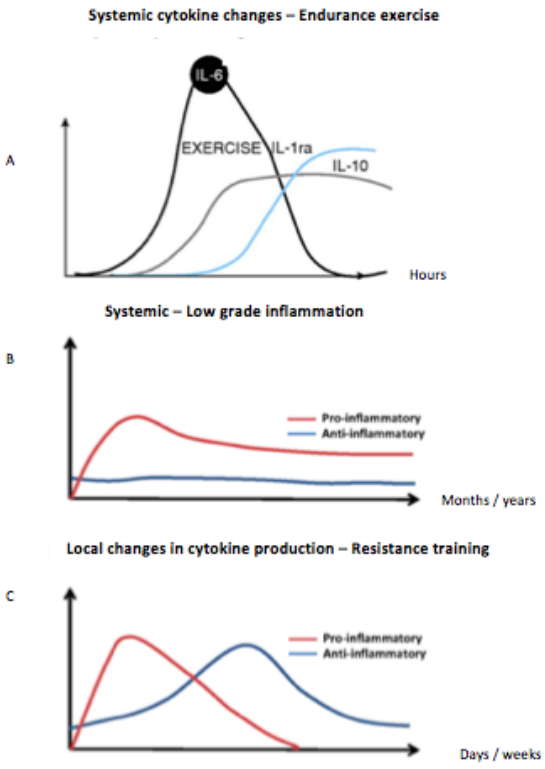

Figure 5. Suggested alterations in systemic and local cytokine appearance during different training/aging scenarios. (**A**) Endurance exercise has been associated with systemic increases in IL-6, rapidly declining again following exercise (hours). (**B**) Systemic low grade inflammation, has been associated with inactivity, ageing and visceral adipose tissue. It is defined as a long lasting increase in systemic pro-inflammatory cytokine concentration (months). (**C**) Local changes in muscle cytokine production following resistance training, has been theorized to be a two sequenced event with increases in pro-inflammatory cytokines prior to increases in anti-inflammatory cytokines. The sequenced response has been suggested to last from several days to several weeks.

Local changes in inflammation after muscle damage have been investigated in several studies. Most of these investigations have been performed in studies with severe muscle damage and tissue regeneration – often combining electric stimulation and eccentric exercise (Saclier et al. 2013; Tidball and Villalta 2010). The local response to injury has been suggested to consist of two waves of inflammatory responses lasting for several days to several weeks. The first sequence is characterized by pro-inflammatory macrophages expressing high amounts of pro-inflammatory cytokines (TNF-α and IL-1b), followed by anti-inflammatory macrophages expressing high amounts of anti-inflammatory cytokines (IL-10 and TGF-β) (Chazaud 2014;

Saclier et al. 2013) (figure 5c). Cytokines expressed by macrophages are believed to play a crucial role in muscle regeneration, playing an active role in monitoring the muscle precursor cell fate, important for effective muscle regeneration (Arnold et al. 2007).

1.3.3 Macrophages – Origin and types

Macrophages constitute a crucial part of the immune system. They secrete various cytokines important for inflammation and protection against bacteria and foreign organisms. Additionally, their potential role as a myogenic precursor cell regulator makes macrophages an interesting topic to investigate further. Macrophages, known for their phagocytosing functions, have extensively been recognized for their central role in the innate and adaptive immune system preventing the presence of pathogens such as invading microbes. Due to this, macrophages have mainly been considered inflammatory and deleterious in the tissue. However, recent studies have shown that macrophages are also involved in tissue repair and regeneration, playing a key role in the orchestration of inflammatory and anti-inflammatory processes resulting in tissue restoration (Benedicte Chazaud 2014; Murray and Wynn 2012).

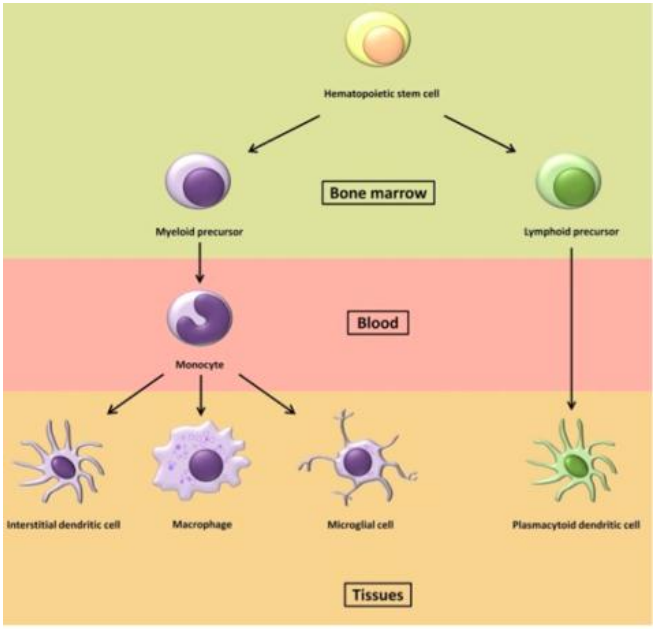

Figure 6. Monocyte macrophage lineage. All cells from the monocyte macrophage lineage appear to derive from the hematopoietic stem cell located in the bone marrow. The hematopoietic stem cell can differentiate into the myeloid precursor cell, which is then able to migrate into the bloodstream and differentiate into the monocyte. Monocyte migration to specific tissues and further differentiation occur upon stimulation from cytokines from the local environment. Illustration from Le Douce (2010).

The term macrophage covers a wide range of cell phenotypes with distinct locations and functions (Kumar and Jack 2006). This thesis will focus on the type of macrophages located in the skeletal muscle. Macrophages in the skeletal muscle are believed to derive from monocytes. These macrophages express self-renewing properties but it is still not known whether the population maintains itself throughout life. Experimental studies with ablation of resident macrophages have demonstrated the capacity of bone marrow derived cells (monocytes) to replace resident cells either by converting themselves or performing a similar role (Davies et al. 2013). The bone marrow contains hematopoietic stem cells with the ability to divide and differentiate into different types of progenitor cells - one being the myeloid cell. The myeloid cells differentiate into the monocyte, which circulates in blood and is the mobile progenitor of the sedentary tissue macrophage (see figure 6) (Murray and Wynn 2012; Mosser and Edwards 2008; Kumar and Jack 2006).

During muscle damage or infection, monocytes are recruited to the site of injury. Monocytes extravasate through the endothelium and enter the tissue where they mature into macrophages and take up residency. The outcome of the maturation depends on the local environment in the tissue turning it into a cell with pro- or anti-inflammatory subsets (Murray and Wynn 2012; Kumar and Jack 2006; Mosser and Edwards 2008). These two opposing isoforms represent the extremes in a spectrum of many possible isoforms. The pro-inflammatory macrophage is also referred to as classically activated macrophage or simply M1, whereas the anti-inflammatory macrophage is known as the alternatively activated macrophage or M2. M2 macrophages can be further divided into three subtypes M2a, M2b, M2c – illustrating the many isoforms. It is worth noting that all macrophage populations have been investigated with well-defined stimuli in in vitro studies - therefore they may not imitate what happens in vivo, where additional stimuli may occur and overlap (Benedicte Chazaud 2014; Mosser and Edwards 2008).

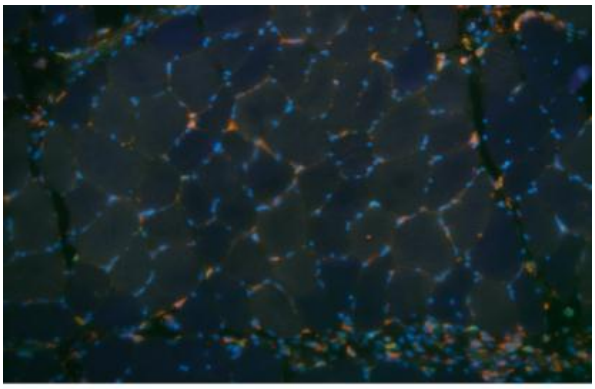

Figure 7. Resident macrophages appear to reside and accumulate in the perimysium. Depicted is a muscle sample from a test subject showing a fascicle surrounded by connective tissue (perimysium). Orange areas represent macrophages which appear to be present in large numbers in the perimysium.

The attraction of monocytes to the site of injury is believed to be orchestrated by the resident macrophages in the muscle tissue. Resident macrophages are accumulated in the perimysium and epimysium (Brigitte et al. 2010; Bénédicte Chazaud et al. 2009) (figure 7) and appear to play an important role for recruitment of neutrophils and monocytes from the circulation. This is emphasized in animal studies where depletion of resident macrophages in injured muscle reduces monocyte infiltration after injury (Bénédicte Chazaud et al. 2009).

When referring to macrophages it is important to keep in mind that the word covers a variety of cells with distinct functional phenotypes determined by the environment in which they reside. Monocytes exposed to environments containing pro-inflammatory cytokines: tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) will drive the activation of the cell towards an inflammatory phenotype. Inflammatory macrophages (M1) have an enhanced microbicidal capacity and secrete high levels of pro-inflammatory cytokines (IL-1 and and TNF-α), reactive oxygen species (Mosser and Edwards 2008; Mantovani et al. 2004) and cytotoxic levels of nitric oxide

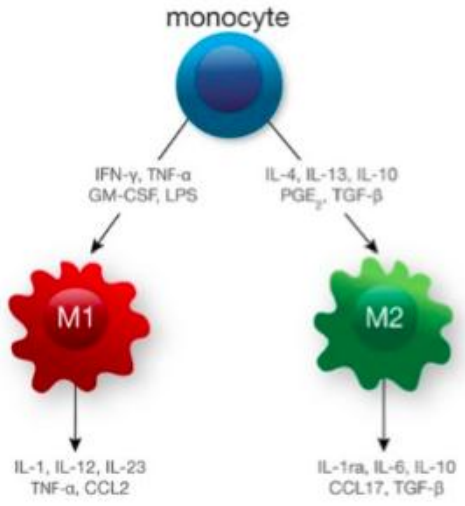

Figure 8. Monocytes from peripheral blood differentiate in response to mediators in tissues, giving rise to pro-inflammatory macrophages (M1) or anti-inflammatory macrophages (M2). Illustration from Brock, Caymanchem.com

(Tidball and Villalta 2010; Mantovani et al. 2004). Nitric oxide is produced by NOS (nitric oxide synthase) enzymes, these enzymes are in turn highly expressed by pro-inflammatory macrophages providing them their ability to produce NO and kill microorganisms (Mosser and Edwards 2008; Murray and Wynn 2012). Some studies have used the expression of NOS in pro-inflammatory macrophages to distinguish and identify these cells in muscle tissue using specific antibodies (Saclier et al. 2013). The pro-inflammatory cytokines produced by M1 macrophages are believed to be an important first component of host defense, also important for removal of cell debris in necrotic muscle fibers. Anti-inflammatory macrophages (M2) are activated primarily by the cytokines IL-10, IL-4 and to some degree IL-6 and are characterized by low levels of pro inflammatory cytokines. The activation of the various M2 macrophages are believed to dampen inflammation, enhance extracellular matrix synthesis and enhance wound healing and tissue repair (Saclier et al. 2013; Mosser and Edwards 2008).

1.3.4 Macrophages following muscle damage

The following chapter highlights the proposed mechanisms of local muscle repair following muscle tissue damage in various injury models. This assignment will primarily focus on local inflammation in relation to resistance training. The local inflammation response to resistance training is characterized by pro-inflammatory cytokines arriving prior to anti-inflammatory cytokines. This was previously illustrated in figure 5c.

Table 1 displays the current available techniques utilized to induce damage for investigation of local muscle injury and local inflammation. The table highlights advantages and disadvantages with each method. Most of our knowledge regarding local inflammation has been obtained in animals studies. It is important to note that it doubtful whether investigations conducted in animal studies also apply to humans; nonetheless, it may provide helpful insight to important mechanisms for future human studies.

Muscle damage models		Advantage	Disadvantage
Physiological damage	Concentric exercise	Easy to perform	minimal/no damage
	Eccentric exercise	Easy to perform	moderate damage, regenerating fibers?
Supraphysiological damage	Electric stimulation + Ecc./Con. exercise	Muscle damage/ regenerating fibers	Applicable to physiological situations ? Difficult setup
Disease		Defect mechanisms, causal explanations	Several mechanisms of interest may be influenced by disease
Animal studies	Partial excision Toxic injections etc.	Allow specific and invasive investigations	Applicable to humans?

Table 1. Available protocols to induce muscle injury and local inflammation in humans. The table highlights the advantages and disadvantages from current damage techniques. As illustrated in the table it is difficult to induce severe damage in human with the current available methods. In animals more invasive and specific investigations are allowed, however, we do not know if studies from animals are applicable to humans.

Animal studies

According to animal studies, neutrophils are the first responders to muscle damage. Neutrophils appear in elevated numbers within 2 hours, peaking 24 hours after damage. Following the onset of neutrophils, pro-inflammatory macrophages invade the muscle reaching significantly elevated concentrations 24 hours post injury continuing to increase in numbers within the first 48 hours after damage (Tidball and Villalta 2010).

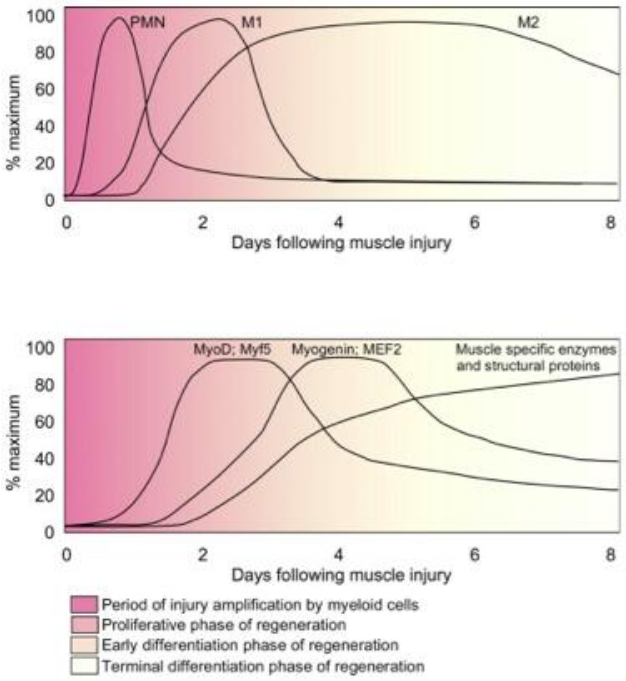

Figure 9. Generalized time course of changes in myeloid cell populations (top) and changes in the expression levels of muscle-specific transcription factors, enzymes, and structural proteins in muscle following acute injury (bottom). PMN: neutrophils, M1: M1 macrophages, M2: M2 macrophages. Illustration from Tidball (2010)

As depicted in figure 9, pro-inflammatory infiltration of the tissue is followed by an increase in anti-inflammatory macrophages reaching a peak concentration around 4 days post injury. These anti-inflammatory macrophages are believed to remain elevated for many days (Tidball and Villalta 2010). A review by Chazaud (Benedicte Chazaud 2015) confirms this timeline on macrophage alterations. Chazaud concludes that the same macrophage kinetics are always observed after muscle damage in animals, but range, duration and presence of the two types of macrophages vary depending on the protocol used. A study in mice by Arnold (Arnold et al. 2007) has suggested that following skeletal muscle damage, macrophages eventually undergo a transition from pro-inflammatory to an anti-inflammatory profile. The cellular and molecular mechanisms responsible for the transition are poorly understood, however studies have speculated that macrophages/monocytes possess a plastic nature capable of converting its isoform.

Based on these findings it is speculated that inflammation undergoes a series of carefully regulated stages to ensure an efficient return to homeostasis. Several studies have shown that interfering with the sequence of macrophage polarization states leads to a defect in muscle regeneration. Especially, it appears to be essential for the pro-inflammatory phase to occur before the switch to an anti-inflammatory phase. This has been investigated by inducing anti-inflammatory cytokines at an early time point after damage or inhibiting anti-inflammatory cytokines at a late time point – both with the result of an impaired muscle regeneration (M Saclier et al. 2013; Chazaud 2014).

Human studies

In humans, a limited number of studies have investigated the behavior of macrophages. Originally intended to investigate the mechanisms of a repeated bout effect, Mackey (Mackey et al. 2011) examined the total number of macrophages in human muscles 48 hours and 30 days after electric stimulation. In this study pro- and anti-inflammatory macrophages were not distinguished but an increase in the total amount of macrophages ($CD68^+$ cells) 48 hours after electric stimulation was observed. 30 days later, the stimulated leg still expressed a significant higher number of macrophages compared to the control leg – illustrating a prolonged macrophage response compared to that just seen in animals. However, since this setup was performed using electric stimulation it is uncertain whether a similar response would occur in humans under physiological circumstances. Another study (Paulsen et al. 2012), investigated inflammatory markers after eccentric resistance training of the elbow flexors. Alterations in macrophages were not the primary outcome; however they observed the highest individual macrophage ($CD68^+$) cell counts at 4d and 7d after the exercise bout in those subjects that were moderately or severely affected by the eccentric training. Again, no differentiation was performed between pro- and anti-inflammatory macrophages. A study by Przybyla investigated macrophages in young and elderly following an acute bout of physiological resistance training of the lower limbs. 3 days after resistance training, no changes were seen in total number of macrophages ($CD68^+$) in either young or old subject. In the young group, however, they found an increase in pro- and anti-inflammatory macrophages, whereas no changes in subtypes were detected in the elderly, suggesting that the changes in young muscle may be caused by resident macrophages and not monocyte infiltration.

A study by Saclier (Saclier et al. 2013) investigated macrophage subtypes in protocols combining voluntary contractions with electric stimulation. 7 days after muscle damage, samples were analyzed for pro- and anti-inflammatory macrophages. The study found that pro- and anti-inflammatory macrophages were present in regenerating areas at the same time challenging the simplistic view that one biological event is associated with one type of macrophages. They claim that pro and anti-inflammatory macrophages coexist in the same regenerative areas; however, when macrophage subtypes were analyzed in relation to the phase of muscle regeneration (early vs. late, using myogenin) pro-inflammatory macrophages were more prevalent in the early regenerative phase whereas anti-inflammatory macrophages were more apparent during the late phase of regeneration. This indicates a shift from one macrophage subtype to another which may overlap spatially and temporally. It is important to keep in mind that this study is very invasive, intended to cause muscle necrosis and muscle regeneration it may therefore not represent what happens under normal resistance training settings where necrotic fibers are unusual (Paulsen et al. 2012).

1.4 Macrophages and their influence on Myogenic precursor cells (MPC)

Cytokines are proteins important for cell communication; they orchestrate the immune system and play an important role for regulation of monocytes into pro or anti-inflammatory macrophages. Additionally, cytokines expressed by macrophages are suggested to regulate the myogenic precursor cell (MPC) fate, playing an important role for muscle regeneration, repair and maintenance. In a study by Arnold (Arnold et al. 2007) MPC were isolated in different media containing macrophages treated with pro inflammatory cytokines (LPS/INF-y) or anti-inflammatory cytokines (IL-4 and IL-10) to investigate their influence on MPC activity. As illustrated in figure 10, MPC's treated in pro-inflammatory medium expressed enhanced growth and proliferation, whereas the anti-inflammatory media increased MPC differentiation and fusion. This suggests that the macrophage activation state monitor the myogenic process (Arnold et al. 2007). This is in line with the previously described human in vivo study by Saclier (Saclier et al. 2013). This study found that pro- and anti-inflammatory macrophages coexisted in regenerating muscle areas. However, they found that early regenerating areas (not expressing myogenin) contained more pro-inflammatory macrophages, whereas late regenerating areas (expressing myogenin) contained more anti-inflammatory macrophages. These findings support the theory that MPC exposure to pro-inflammatory cytokines stimulates initial expansion and proliferation, followed by exposure to anti-inflammatory cytokines enhancing differentiation and fusion important for optimal repair.

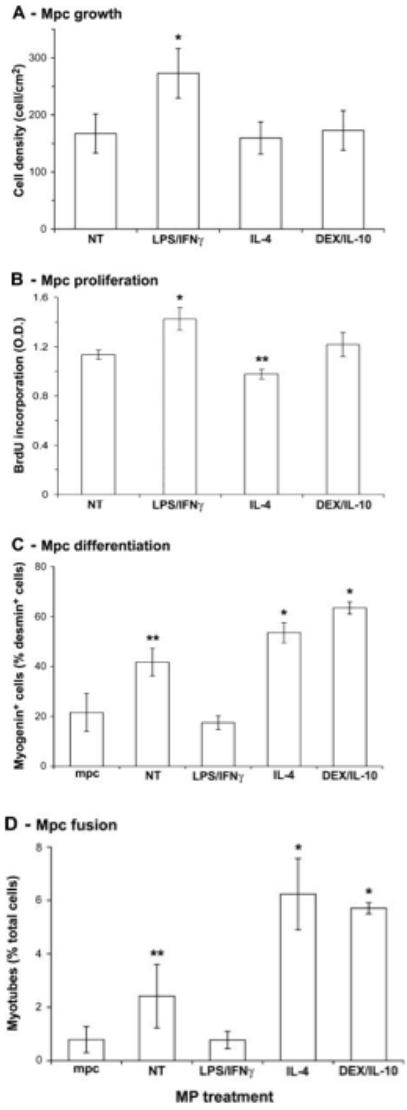

Figure 10. Effects of activated macrophages on MPC fate. MPCs were co-cultured with untreated (NT), LPS/IFN-γ, IL-4, and DEX/IL-10 treated macrophages and analyzed for (**A**) growth (**B**) proliferation (**C**) differentiation (**D**) fusion. Cells were analyzed at day 3 after co-culture, except for BrdU incorporation – monitored during 24 hours of co-culture. Results are mean ± SEM of three experiments. * ($P < 0.05$). Illustration from Arnold (2007)

One way the orchestration of the pro- and anti-inflammatory response is regulated could be the transformation of macrophages from one phenotype to another. The previously mentioned study by Arnold (Arnold et al. 2007) found that pro-inflammatory macrophages decreased their TNF-α secretion and increased their transforming growth factor beta 1 (TGF-β1) secretion following phagocytosis of necrotic muscle cells, suggesting a switch from a pro to an anti-inflammatory phenotype (Arnold et al. 2007). The exact role of TGF-β1 is not well known, but TGF-β1 expression has been closely related to anti-inflammatory macrophages and tissue regeneration (Benedicte Chazaud

2015; Arnold et al. 2007). Increased levels of TGF-β1 are seen 2 days after injury and are maintained throughout the regeneration process. Some studies propose that TGF-β1 downregulates inflammatory TNF-α cytokine production and stimulate cell survival, fibrogenesis and extracellular matrix remodeling (Lemos et al. 2015; Tonkin et al. 2015), whereas other theories suggests that increased levels of TGF-β inhibit satellite cell activation and leads to the formation of fibrotic tissue in response to skeletal muscle injury (Burks et al. 2011). These actions propose TGF-β to contribute to tissue repair, which under ideal circumstances leads to the restoration of tissue, however excessive levels may lead to excess tissue fibrosis and low muscle quality (Leask and Abraham 2004).

1.5 Inflammation in the aging muscle

Macrophage infiltration is a natural process occurring in response to muscle damage or injury. Through the expression of various cytokines, macrophages provide important cues to the orchestration of growth, proliferation and fusion of muscle precursor cells contributing to muscle regeneration. Besides playing a major role in MPC fate, pro and anti-inflammatory cytokines also contribute to changes in the systemic inflammatory profile. Especially in elderly, heightened systemic inflammation has been associated with muscle loss (Degens 2010; Przybyla et al. 2006; Garatachea et al. 2015; Merritt EK et al. 2013). A study by Merritt suggests that impairment in the ageing muscle is a result of exposure to a heightened and prolonged pro-inflammatory signaling that disrupts the local environment leading to failed myogenesis. This study found that several pro-inflammatory cytokines including TNF-α and IL-6 increased in ageing humans (Merritt EK et al. 2013). Additionally, Merritt found that nuclear factor kappa B cell (NF-κB) increased in elderly. NF-κB is a transcription factor closely related to the pro-inflammatory cytokine TNF-α. The NF-κB signal pathway has been linked to a loss of skeletal muscle mass in various disease and immobilization scenarios; however its relation to Sarcopenia has not yet been confirmed. NF-κB leads to muscle protein degradation, it enhances pro-inflammatory cytokines and blocks the regeneration of myofibers after injury (Li, Malhotra, and Kumar 2008). Separate studies by Visser and Bautmans have shown that increased systemic cytokine production is associated with loss of muscle mass and strength in elderly (Visser et al. 2002; Bautmans et al. 2005). Visser found that elderly (aged 70-79) with increased levels of TNF-α and IL-6 had a smaller muscle mass and strength compared to those with low levels of both cytokines. He also found that pro inflammatory cytokine levels were inversely correlated to muscle mass and strength, suggesting that higher pro inflammatory cytokine levels may contribute to Sarcopenia. In line with Visser, Bautmans found that skeletal muscle function correlated negatively with circulating pro-inflammatory cytokines (Bautmans et al. 2005). He did not see any correlation between TNF-α and muscle loss, but found that circulating Il-6 and CRP were related to muscle mass and strength loss. Altogether it seems that increased levels of pro-

inflammatory cytokines contribute to age related muscle loss. The mechanisms, however, have not yet been clarified.

1.6 Physical activity and inflammation

Exercise cannot reverse the aging process; however it can attenuate some of the deleterious systemic and cellular effects. A large number of studies recruiting elderly have shown clear effects of resistance training on muscle strength and power. A number of studies have also shown an increase in muscle mass in elderly, but these results are less convincing (Garatachea et al. 2015). As previously illustrated (figure 5a), endurance training is believed to markedly increase the presence of systemic anti-inflammatory cytokines IL-6 and IL-10. Both of these are believed to exert inhibitory effects on pro-inflammatory cytokines: IL-1 and TNF-α expression (Petersen and Pedersen 2005), thereby contributing to the suppression of a potential systemic low grade inflammation.

Compared to systemic inflammation and systemic inflammation markers, little is known about local tissue inflammation in response to resistance training and ageing. A study by Levinger has shown that a 10 week resistance training protocol did not influence the inflammatory profile or CRP levels in elderly (age 50) (Levinger et al. 2009). A longer intervention period has been proposed a necessity to alter the inflammation profile; however, it is unclear whether improved CRP levels in response to resistance training simply reflect reductions in fat mass. Greiwe et al. has shown that inflammation and the inflammatory response may be increased and dysfunctional in aged skeletal muscle (Greiwe et al. 2001). The study found that TNF-α was transcribed by young and old human myocytes and that TNF-α expression was increased in the muscle of frail elderly compared to young individuals. Additionally, 3 months of resistance training caused a decrease in muscle TNF-α compared with the control group (Greiwe et al. 2001). In general, it remains unknown whether systemic inflammation correlates with observed local inflammation. Though speculative, this may indicate that it may be necessary to distinguish local from systemic inflammation. Even though some cytokines may play an important role in both scenarios the direct link between systemic inflammation and local inflammation remains to be elucidated.

Most of our knowledge regarding inflammation and its role in skeletal muscle repair has been derived from human or animal studies with severe damage protocols. These protocols provide insight to mechanisms important for muscle regeneration, but may not imitate damage responses induced by physiological damage. As discussed, macrophages cover a wide variety of cells with distinct functional isoforms. Depending on their isoform they express pro-inflammatory and anti-inflammatory cytokines important for regulation and orchestration of myofiber formation/repair. It seems that the timing and type of cytokines expressed by macrophages is a crucial factor for successful regeneration of muscle. Also, a large number of studies

investigating the ageing human muscle have associated heightened levels of certain pro-inflammatory cytokines, similar to those expressed by macrophages, with impairments in muscle quality and muscle mass. These findings make macrophages an interesting target to investigate further. Little is known about the presence of macrophage subtypes in skeletal muscle and what constitutes an appropriate inflammatory response to damage. Such investigations may contribute to our understanding of inflammation and the ageing muscle.

The purpose of this assignment is to achieve a method to detect macrophage content in tissue and apply it to investigate local changes following an acute and chronic resistance training protocol in elderly. So far, only a minimum of studies have investigated local tissue inflammation following a physiological bout of exercise in humans, which could be ascribed to a lack of reliable methods to detect macrophage subpopulations. Animal studies have suggested a timeline for the orchestration of macrophage infiltration, however we do not know if a similar infiltration is applicable to human tissue. Additionally, it is uncertain how a prolonged resistance training protocol influence local inflammation profile in muscle tissue and whether these potential changes can be detected.

1.7 Thesis statement

The primary purpose of this assignment is to investigate the local macrophage response after an acute unaccustomed bout of exercise and after a 12 week training intervention and further to develop a method for detection of macrophage subpopulations.

1.8 Hypothesis

It is hypothesized that macrophages infiltrate local tissue following an acute bout of heavy resistance training. The local response is characterized by an early infiltration of pro-inflammatory macrophages followed by an infiltration of anti-inflammatory macrophages a few days later. It is hypothesized that young muscles contain a more anti-inflammatory environment compared to muscle in elderly prior to 12 weeks of resistance training. Furthermore, 12 weeks of resistance training is theorized to alter the local inflammatory profile in young and elderly muscle expressed by an accumulated number of anti-inflammatory macrophages compared to baseline. For the detection of macrophages we hypothesize that co-labelling the CD68 antibody with either TNF-α or iNOS will serve as a potential marker for detection of pro-inflammatory macrophages, whereas CD68 combined with either CD206 or CD163 antibodies will function as a reliable marker for detection of anti-inflammatory macrophages.

2 Materials and Methods

Studies included

To test the hypothesis, a study named: counteracting inflammatory muscle atrophy (CIMA) was initiated at Bispebjerg Hospital. However, because of a delayed recruitment of test subjects in the CIMA study, samples from 2 previous studies performed at ISMC were analyzed instead.

The two following studies, an acute study and a training study, were chosen based on their design protocol. The protocol from each of these studies allow me to test my hypothesis, without analyzing data from the CIMA study. Both studies had a different emphasis in their original edition; however this should have no influence on the findings in this assignment.

2.1 Study 1 – acute study

This acute study was originally performed to test the influence of Angiotensin II receptor blocker (Losartan) on a strength training response. It was recently carried out and is therefore unpublished. This study is, due to the setup, well suited for investigations of changes in local inflammation after a heavy bout of unaccustomed exercise. The following describes the main characteristics of the study, from which biopsies have been received. Particularly, details of relevance to test the hypothesis described in this assignment have been included.

Participants

27 old (age 64-90 years) healthy volunteers were recruited for this study and assigned to either a placebo group or a group consuming Losartan. Inclusion characteristics are illustrated in table 2. Distribution of participants into the two groups was done in a double blinded manner with the placebo group containing 14 test subjects and Losartan group containing 13 test subjects. Test subjects had to be non-smokers with no hyper- or hypotension. Additional exclusion criteria were: medicine on a regular basis, cancer, connective tissue diseases or kidney problems. Only sedentary or moderately trained individuals were included in the study. Also, subjects participating in strength training or any other physical training activity on a daily basis prior to the study were excluded.

Table 2. Inclusion characteristics. Baseline characteristics (Mean ± SD) of subjects in the placebo- and Losartan group.

	Placebo (n = 14)	**Losartan** (n = 13)
Age, years	70.8 ± 8.4	69.7 ± 4.0
Height, cm	179.9 ± 5.1	178.8 ± 3.8
Weight, kg	85.7 ± 8.7	75.8 ± 7.6
Body mass index, kg/m2	23.8 ± 2.1	21.2 ± 2.1

Study design

The acute study design is illustrated in figure 11. Losartan or placebo consumption started 10 days prior to an exercise bout and continued until the day when the last biopsy was taken (18 days later). Participants performed a 1 repetition maximum test (1RM) and a heavy bout of resistance training of the thigh muscles of one leg. The 1RM and resistance training protocol was performed in a knee extension device adjusted to each individual (Rehabilitation device, Technogym, Italy), with hips and knees flexed to 90 degrees.

On test day (day 0) people came in and performed their 1RM test. The results of the 1RM test were used to determine the load of the following resistance training. Resistance training consisted of 5 sets of 12 repetitions (70% of 1RM) of active concentric contractions followed by 4 sets of 6 repetitions (110% of 1RM) of active eccentric contractions.

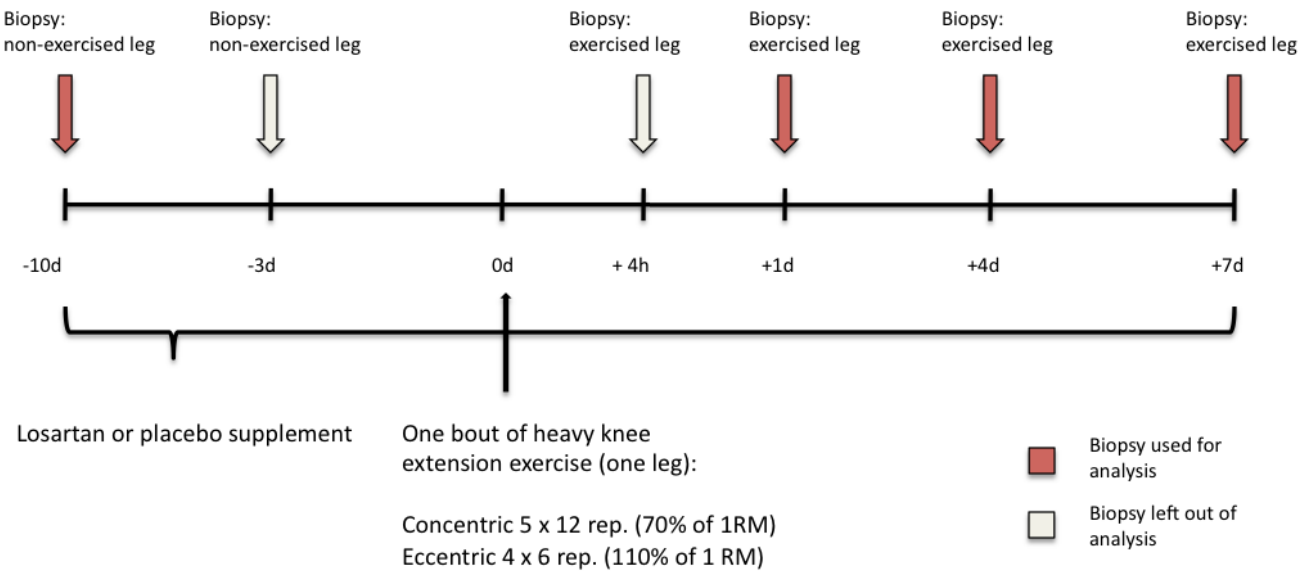

Figure 11. Study protocol from the acute study. The biopsy extracted at -10d serves as the PRE reference sample in my assignment. Additional time points of investigation were 1 day, 4 days and 7 days. Biopsies extracted at -3 days and +4 hours were left out of the analysis

6 muscle biopsies were collected from each test subject over the course of 18 days (see figure 11). All biopsies were collected from the vastus lateralis of either the exercised leg or non-exercised leg. The first biopsy (-10d) was collected 10 days prior to the resistance training (day 0) before ingestion of Losartan or placebo – it therefore serves as a baseline test. The second biopsy (-3d) was collected 3 days before resistance training (week 0) and indicates the potential effects of losartan on resting muscle. These biopsies

were both collected from the non-exercised leg. After completing the resistance training (week 0) biopsies were collected from the exercised leg at the following timepoints post exercise: 4 hours (+4h), 1day (1d), 4 days (4d) and 7 days (7d). These biopsies were all used for detection of potential changes caused by the strength training bout.

2.2 Study 2 – Training study

This training study was originally performed to test the influence of vitamin D intake during resistance training. In my investigations the vitamin D group has been left out, analyzing only the placebo groups. This setup allows for investigation of potential changes in inflammatory content before and after a 12 week training intervention. Main characteristics of the study have been described in the following. Particularly, details of importance to this study have been included whereas some information has been left out playing no role in the investigations performed in this assignment. Additional information about this study can be found in Agergaard et al. 2015.

Participants

Healthy sedentary young (aged 20-30 years) and elderly (aged 60-70 years) men living in Copenhagen, Denmark were recruited. Subjects were excluded if they had participated in resistance exercise during the preceding 6 months, if they had a body mass index (BMI) < 18 or > 30 or if they had illnesses that could affect the musculoskeletal system.

Table 3. Inclusion characteristics. Baseline characteristics (Mean ± SD) of young and elderly subjects in the placebo group.

	Young men placebo (n = 10)	Elderly men placebo (n=10)
Age, years	22.4 ± 1.8	66.6 ± 4.2
Height, cm	181.1 ± 5.5	178.8 ± 6.7
Weight, kg	75.6 ± 9.1	80.4 ± 9.3
Body mass index, kg/m2	23.0 ± 2.3	25.1 ± 1.1
CSA, cm2	59.0 ± 4.8	47.0 ± 7.3
Isometric muscle strength, Nm	209.5 ± 30.5	168.3 ± 35.3

20 young and 20 elderly men were included in the study. Participants were randomized to either a vitamin D group or placebo group. Young and elderly were randomized separately in an allocation ratio 1:1 for the two groups. 6 participants (3 young and 3 elderly) dropped out, but as they were all from the vitamin D-group this will be of no importance for my analysis which is restricted to only the placebo group.

Baseline characteristics for all 20 (10 young and 10 elderly) participants allocated to the control group is shown in table 3.

Training protocol

Participants were subjected to 36 supervised training sessions (12 weeks with 3 sessions/week) consisting of 5-10 minutes warmup on cycle ergometers followed by resistance training exercises of the lower extremities

performed in knee extension and leg press devices. Progressive loading levels were monitored and adjusted continuously throughout the entire training period. 1 repetition maximum (1RM) was estimated from a 5 repetition maximum test – performed every two weeks to ensure that training load was relative to the strength progression throughout the training period.

During the first 6 training sessions, participants completed 3 sets of 12-15 repetitions at 65-70 % of 1 RM. During sessions 7-12, participants performed 3 sets of 10-12 repetitions at 70-75 % of 1 RM, increasing to 4 sets at 70-75 % of 1 RM during session 13-18. From session 19 and on, participants performed 5 sets with training load progression from 8-10 repetitions at 75-80 % of 1 RM in session 19-27 to 6-8 repetitions at 80-85 % of 1 RM in session 28-36. Exercises were performed in a moderate slow, controlled manner with 1-2 s in the concentric- and eccentric phase with a rest of 1-3 min between sets.

Table 4. Training protocol Agergaard et al.

Training session	sets	reps	% of 1 RM
1 - 6	3	12 - 15	65-70
7 - 12	3	10-12	70-75
13 - 18	4	10-12	70-75
19 - 27	5	8-10	75-80
28 - 36	5	6-8	80-85

Original study design and biopsies

A biopsy from the lateral part of the m. vastus lateralis muscle was obtained before the training started (week 0) and after the last training session (week 12). At week 12, two biopsies were taken 4 h (TR+4h) and 48 h (TR+48h) after the last exercise session. Biopsies obtained before the training intervention (week 0) were randomly chosen from either the left or the right leg. Biopsies at week 12 (TR+4h) were taken from the contralateral leg to the one chosen at week 0, whereas the biopsy at 48 hours after last training (TR+48h) was taken from the same leg as the biopsy before the training intervention (week 0). The isometric strength tests performed before and after the intervention were carried out at time points with no conflicting influence on muscle samples and inflammation. Figure 12 illustrates the protocol for a given subject in the study by Agergaard et al. (Agergaard et al. 2015)

Biopsies used for this study

The biopsy performed +48h after intervention stop was left out of my analysis. The biopsy at week 0 serves as a baseline sample (PRE) and the 12week+4h biopsy as the post intervention sample (POST). Possible changes in PRE and POST biopsies are interpreted as beneficial effects of the training intervention.

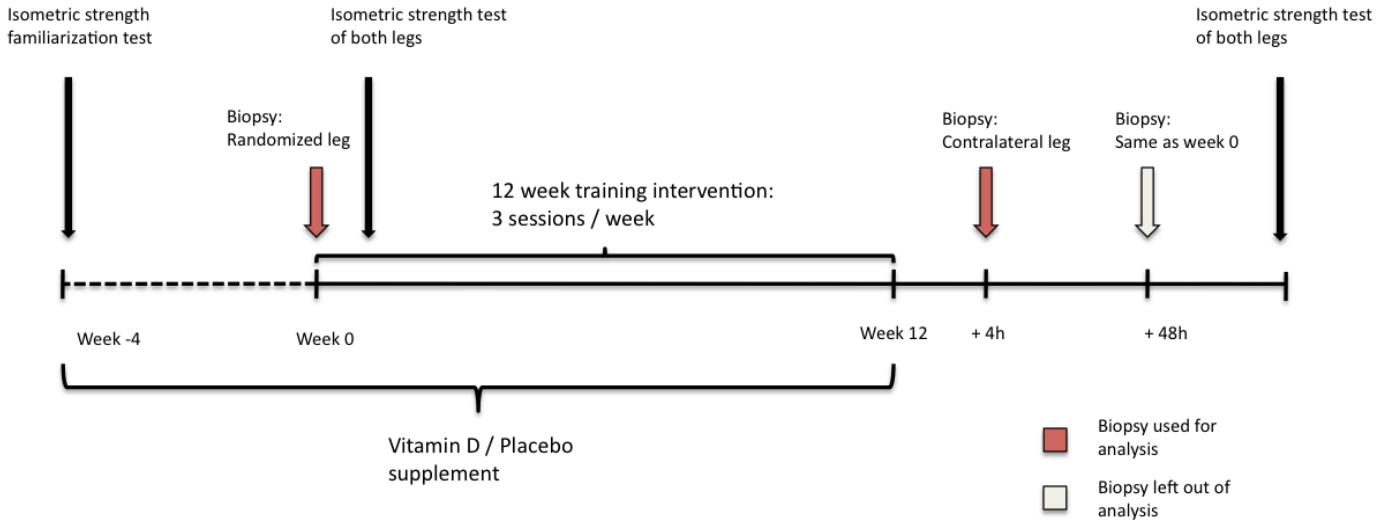

Figure 12. Study protocol from the training study. The biopsy at week 0 serves as a baseline sample (PRE) and the 12 week + 4h sample as the post intervention sample (POST). The biopsy at 12 weeks + 48h was excluded from the analysis.

2.2.1 Biopsy procedure

All muscle biopsies at ISMC are collected with similar procedures (Agergaard et al. 2015;Saclier et al. 2013; Mackey et al. 2011). Since biopsies to test my hypothesis are acquired from studies conducted prior to my arrival at the institute, I did not assist in the gathering of these specific muscle samples. However, my participation in the CIMA study, originally intended to test my hypothesis, has provided me numerous opportunities to assist on biopsy collection and knowledge on the following technique.

Samples were collected from the m. vastus lateralis under local anesthesia (1% lidocaine). A small incision was made prior to the insertion of a 4-mm Bergstrom needle (Bergström, Stockholm, Sweden) with manual suction. After collection, the biopsy samples were freed from visible blood, fat or connective tissue - fibers were then aligned and embedded in Tissue-Tek (Sakura Finetek Europe, AJ alphen aan den rijn, Netherlands). Samples were frozen by immersion in isopentane, precooled by liquid nitrogen and stored at -80^{o} C until further analysis.

When all biopsies were collected and ready for further analysis, serial transverse sections (10 um) were cut from the muscle specimen mounted in Tissue-Tek in a cryomicrotome (HM 560, Microm, Germany) at -24 degrees. During this process cross sections were transferred onto SuperFrost Plus glass slides (Menzel Gläser, Braunschweig, Germany).

2.2.2 Immunohistochemistry

Immunohistochemical staining was performed one study at a time. Initially, sections were allowed to dry for 30 min. When dry, they were encircled with a water repellent Dako pen (Agilent technologies, US) and fixed in Histofix (Histolab, Sweden) for 10 minutes. Sections were then washed twice in TBS and incubated overnight in a 1% BSA (Bovine serum albumin) buffer mixed with two primary antibodies at 4°C. Double staining was necessary to determine macrophages and their subtype. Primary antibodies for evaluation of anti-inflammatory macrophages were CD68 (HPA048982, rabbit) and CD163 (SC-20066, mouse) both in a 1:200 solution with the BSA buffer. The next day sections were washed twice in TBS and incubated for 45 minutes in two secondary antibodies mixed in a 1% BSA buffer. Secondary antibodies were Flour goat anti-rabbit 568 (A-11036) and Flour goat anti-mouse 488 (A-11029) both in a solution 1:500 with the BSA buffer. Mounting medium (Prolong gold, Molecular probes) containing 4',6-diamidino-2-Phenylindole (DAPI) was used to stain the cell nuclei blue.

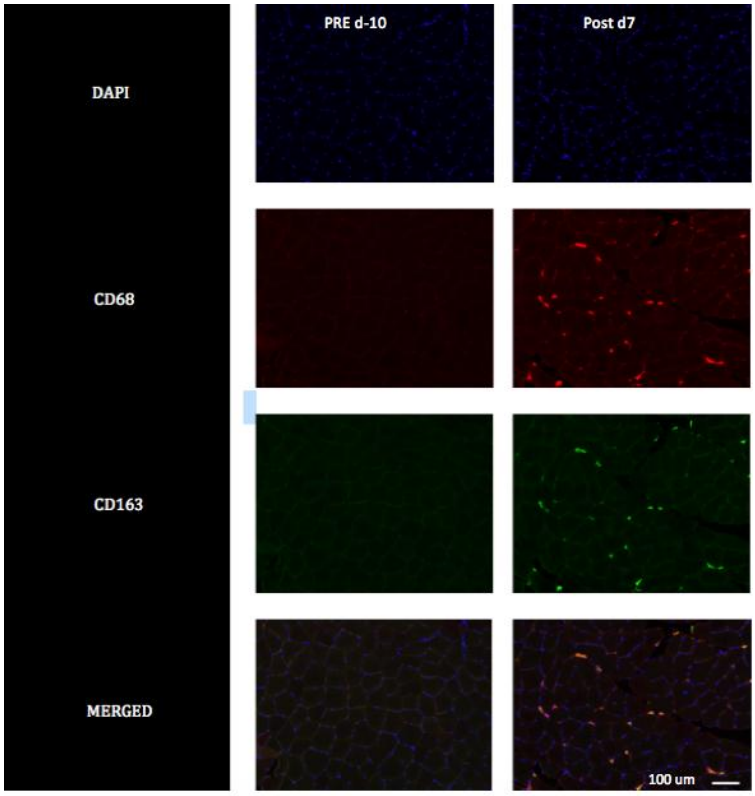

Figure 13. Immunohistochemical staining. Pre (day -10) and post (day 7) images from a subject performing a single bout of heavy unaccustomed resistance training. DAPI, CD68 and CD163 antibodies were chosen based on images from test samples analyzed prior to this study.

All sections from the chronic adaptation study (Agergaard et al. 2015) were stained in the same mix of primary and secondary antibodies. Samples from the acute study had to be separated into two equal sized portions, due to a larger number of slides. The previously described protocol was then carried out twice with identical solutions of primary and secondary antibodies for both protocols.

For further descriptions on groundwork for selection of antibodies see methodological considerations.

2.2.3 Image acquisition and analysis

Images were captured using an Olympus BX51 fluorescent microscope (Olympus Hamburg, Germany) with an Olympus DP71 digital camera conducting images from the microscope to a high resolution computer screen using the software Olympus CellSens standard (version 1.14, Olympus corporation). Images were captured with a 10x objective in a dark room at normal temperature. Macrophage staining was assessed from

2 nonoverlapping images of 882 x 663 microns, estimated to contain 200 muscle fibers. This procedure was chosen to avoid areas of perimysium as these areas contain large numbers of resident macrophages. Therefore, aligned fibers, covering the entire width and height of the image and areas containing no or little perimysium were criteria for area selection on biopsies. If two areas meeting all criteria did not appear, the best possible areas fulfilling the criteria were chosen. Three images of the same area were collected, representing each of the fluorescent colors used to analyze samples (DAPI-blue, CD68-red, CD163-green) – images were then later merged into one image consisting of the three layers. To avoid bias, areas were selected from DAPI staining, followed by the images of CD68 and CD163 associated with that area.

Images were merged using ImageJ (version 1.50B – www.fiji.sc) and then blinded (for time and drug) prior to analysis by assigning each picture a randomized combination of numbers and letters.

Picture analysis was carried out using ImageJ (version 1.50B –www.fiji.sc) using the cell counter plugin (Kurt de Voss, Sheffield University). Image area was measured and number of muscle fibers was counted for all images. Additionally, all images were counted for anti-inflammatory macrophages $CD163^{+}/CD68^{+}$/DAPI, macrophages that did not express the anti-inflammatory marker $CD163^{-}/CD68^{+}$/DAPI , and cells other than macrophages expressing the anti-inflammatory marker $CD163^{+}/CD68^{-}$/DAPI.

Figure 14 illustrates how macrophages were counted. Cell nuclei (DAPI) had to be placed centrally in CD68 and CD163 expression. To estimate location of the cell nuclei during analysis, three layers of images (DAPI, CD68, CD163) were merged with the option of removing or adding layers. Cells expressing CD68 and CD163 were counted as anti-inflammatory macrophages whereas cells expressing CD68 but not CD163 were counted as macrophages that were not anti-inflammatory. Due to a lack of better options (see methodological considerations) macrophages that did not express anti-inflammatory markers were treated as pro-

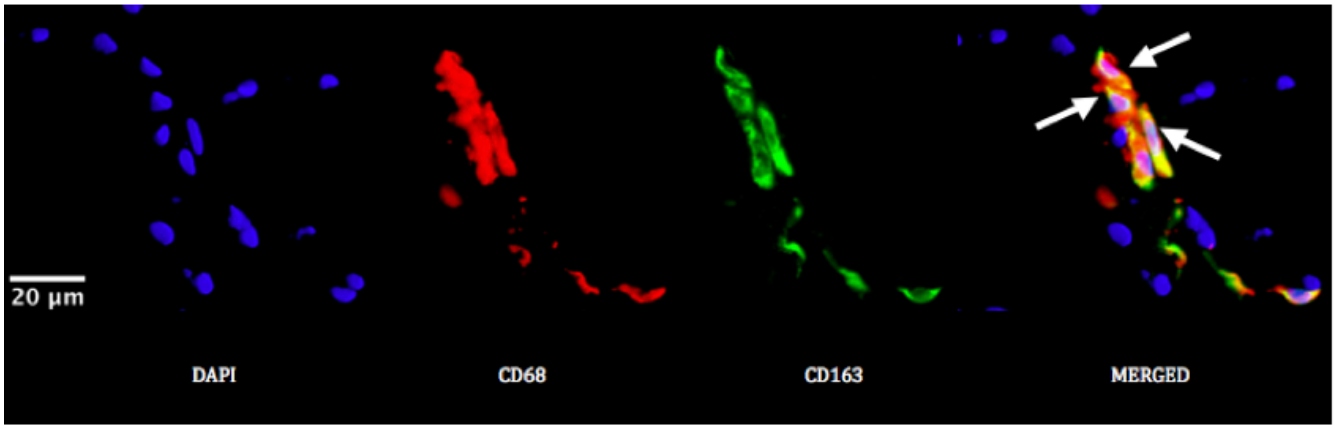

Figure 14. Identifying macrophages. Cell nuclei were stained using a DAPI antibody, macrophages using a CD68 antibody, and anti-inflammatory macrophages using CD163 antibody. Images were merged for analysis. Cells located centrally in CD68 expression only were identified as macrophages. Cells located in CD68 and CD163 expression were counted as anti-inflammatory macrophages (arrows).

inflammatory macrophages. This method, however, will not provide the absolute picture and is intended only as a rough estimate of events. All calculations performed for anti-inflammatory macrophages ($CD163^+/CD68^+$/DAPI) were also carried out for macrophages that did not express the anti-inflammatory marker ($CD163^-/CD68^+$/DAPI). The latter is the one now considered as a rough estimate for pro-inflammatory macrophage activity. Observing the $CD163^+/CD68^-$/DAPI activity was used as a measure for the specificity of the CD163 – revealing whether it binds to cells other than macrophages. Once image analysis was complete, images and associated data were unblinded for statistical analysis.

2.3 Data analysis

The results from the acute study and the training study were analyzed separately. The acute study consisted of 27 subjects (n = 27). 13 were assigned to a Losartan group, whereas 14 were assigned to a placebo group. One pre biopsy from a subject in the Losartan group was excluded due to its poor quality and anomaly, deviating significantly from all other biopsy samples to that time (Losartan, pre n = 12) .

The training study consisted of 10 young and 10 old subjects (n=10). Similar to the acute study, one pre biopsy from the young group was excluded due to poor biopsy quality with fibers impossible to count (young pre, n = 9).

In both studies, cells were analyzed as CD68+ cells, CD68+CD163+ cells, CD68+CD163- cells, representing total number of macrophages, anti-inflammatory macrophages and pro-inflammatory macrophages respectively (see methodological considerations). Cell counts from images representing each individual biopsy were added together to get the total amount of CD68+ cells, CD68+CD163+ cells, CD68+CD163- cells for each biopsy. The sum of each cell type was then divided by total area and total fiber count to get an expression of cell/fiber and cell/mm^2 for each biopsy sample. These calculations were carried out in Windows Excel and performed for all subjects and all time points.

2.4 Statistical testing

Data (cell/fiber and cell/mm^2) was tested for normal distribution using a graphic approach and a normality test (Shapiro-Wilk, SigmaPlot ver. 13.0). Normal distribution is necessary to meet the assumptions of parametric statistical tests. Since data did not fulfill the requirements associated with a normal distribution, data was transformed using a logarithmic transformation. Transformed data was inserted to SigmaPlot (SigmaPlot ver. 13.0, Systat Software, USA) performing a two-way repeated measures ANOVA test.

In the acute study, subjects were tested for drug (, Placebo), time (PRE ,1D, 4D, 7D) and interaction (drug x time). The primary interest of this study was to investigate changes over time. Since no significant interaction effect were found for DRUG x TIME in any analysis, results from the Losartan and Placebo

group were pooled into one group containing all 27 subjects (results for individual groups are shown in appendix 1).

In the training study, data was log transformed and two-way repeated measures ANOVA test was performed for factors: Age (young, old), time (Pre, Post) and interaction AGE x TIME.

In both studies, results were treated as significant if $P < 0.05$ and a Student-Newman-Keuls multiple comparison procedure was used as a post hoc test to identify sample means significantly different from each other. Data are presented as geometric mean ± back transformed standard error mean (SEM) unless otherwise indicated.

2.5 Methodological considerations

2.5.1 Choosing antibodies

In order to detect changes in muscle inflammation, being able to distinguish between pro and anti-inflammatory macrophages is of great importance. Many studies distinguishing between pro- and anti-inflammatory macrophages have done so in animal studies, where antibodies used for macrophage staining are relatively well known, however, antibodies in humans differ from those in animals. CD68 has been widely accepted as a marker expressed on macrophages and monocytes in humans, whereas CD163 and CD206 are associated with the anti-inflammatory subtype of macrophages, also in humans. Antibodies for these markers were assessed on test sections before applying them onto my actual biopsy samples. Initially, a CD68 antibody control test was performed using two different primary antibodies for CD68 staining (DAKO M0718, mouse, 1:500 & Sigma HPA048982, rabbit, 1:200) showing similar results. When determined on a primary CD68 antibody, this was then tested in combination with a primary antibody for CD206 (ab8918, mouse, 1:200) and later a primary antibody for CD163 (SC-20066, mouse, 1:200) to determine their specificity as subtype markers. In agreement with literature they both showed similar activity when analyzed with a microscope, therefore choosing one over the other would have no significant impact on results.

Choosing an antibody to determine the pro-inflammatory macrophage subtype, however, proved to be more difficult, as staining of pro-inflammatory macrophages is still poorly understood. Several studies and reviews by Mosser (Mosser and Edwards 2008), Chazaud (Benedicte Chazaud 2014) and Mantovani (Mantovani et al. 2004) have associated pro-inflammatory macrophages with cytokine TNF-α and iNOS - produced in the macrophage. Studies performing subtype staining of proinflammatory macrophages is, however, extremely limited. One study by Saclier (Saclier et al. 2013) used iNOS as a proinflammatory marker, her staining

though was performed on samples with regenerating areas not expected to be seen in samples included in the present thesis.

Several experiments with TNF-α and iNOS were performed on test sections in order to investigate their function as markers of inflammatory macrophages. Results are illustrated in figure 15. In the initial staining protocol anti-TNF-α (Abcom, AB34674, rabbit, 1:500) was used as a primary antibody on 5 samples. As depicted in figure 15a, this staining (red) reacted with muscle fibers and connective tissue making it impossible to detect macrophage subtypes, in contrast to the CD68 (green) antibody illustrated in the same row. Same thing was seen when using a iNOS antibody (chemicon, Ab5384,rabbit, 1:500) illustrated in figure 15b – again making it impossible to separate cells expressing iNOS from those that do not. Two new protocols were then tested using the same primary antibodies (iNOS and TNF-α) this time combined with goat serum to block background staining. This protocol did downgrade activity in the muscle fibers, but activity around the fibers still did not allow for analysis (figure 15c). A similar protocol was then tested on

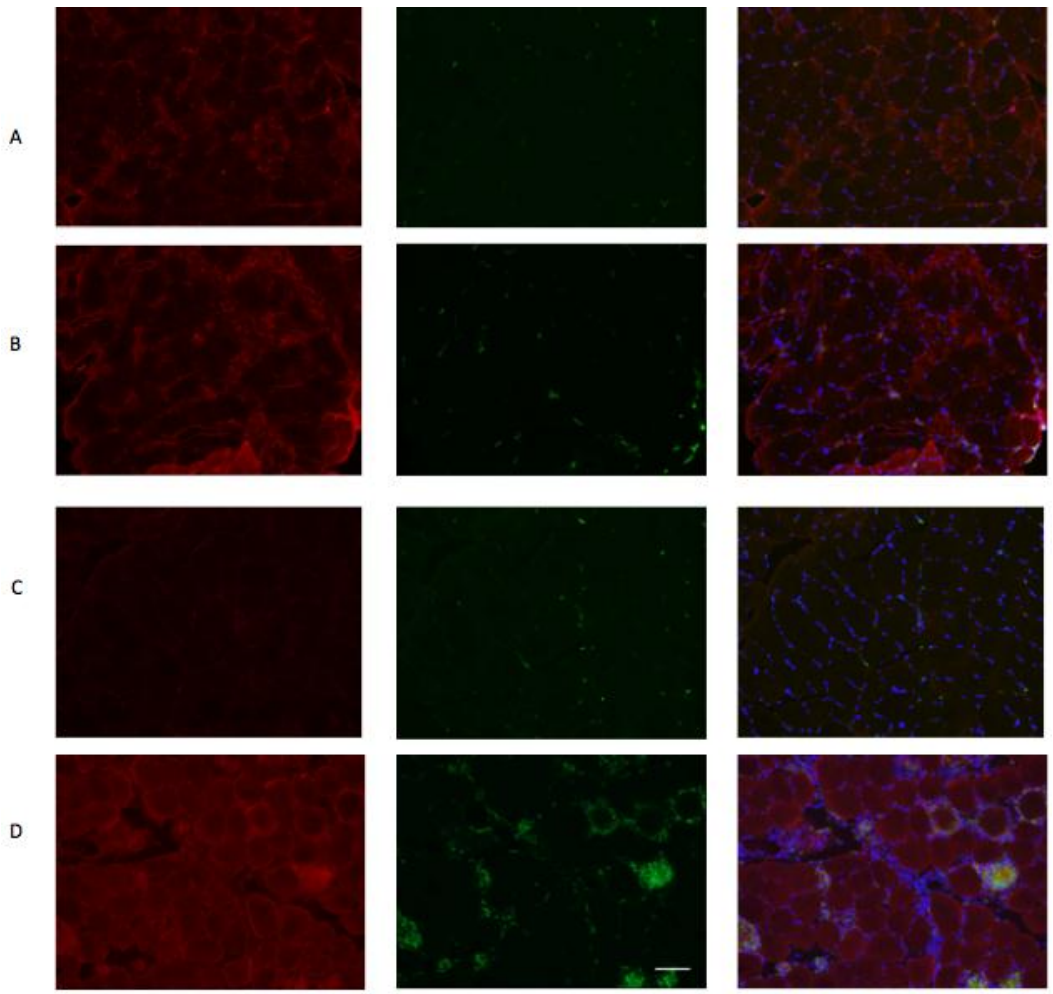

Figure 15.
Identifying the pro-inflammatory macrophage subtype. No successful staining protocol was developed using iNOS (red) or TNF-a (red). Scale bar in (**D**) corresponds to 100 um

(**A**) TNF-a, CD68, merged images
(**B**) iNOS, CD68, merged images
(**C**) TNF-a + goat serum, CD68, merged images
(**D**) TNF-a + goat serum, CD68, merged images.

sections from a study by Saclier (Saclier et al. 2013), who induced severe muscle damage using electric stimulation. As no previous work, has investigated macrophage subtype alterations following a physiological bout of resistance training, we could not rule out the theory that pro-inflammatory macrophages are unaltered following exercise with no myonecrosis/regenerating fibers, explaining why we could not detect any pro-inflammatory macrophages in our immunohistochemical images. We involved the samples from Saclier to assure that we investigated sections that contained pro-inflammatory macrophages with certainty. Still however, we were not able to come up with a result that provided clear images (Figure 15d). We tested one additional protocol using another anti- TNF-α antibody (abcam, ab6671,rabbit, 1:100 – 1:1000) – but just like the others we were unable to see good immunohistochemical images.

Identifying the suitable antibodies for immunohistochemical staining of certain macrophage subtypes in muscle tissue proved to be a difficult task. We were unable to develop a method to detect cells expressing iNOS or TNF-α, believed to be associated with proinflammatory macrophages. Due to these difficulties analysis was performed using only the CD68, CD163 and DAPI staining. Cells expressing all 3 markers were counted as anti-inflammatory macrophages whereas cells that did not express CD163 but CD68 and DAPI were counted as pro-inflammatory macrophages. This method does not illustrate the exact circumstances and should be interpreted with care. However, it will serve as an indicator – and are believed to provide more accurate and reproducible results than an analysis involving any of the proinflammatory antibodies could have.

2.5.2 Fiber count

Prior to analysis, all biopsies were screened for size, fiber alignment and content of perimysium. This screening was performed to estimate a number of fibers that could be analyzed in all biopsy samples. Due to individual variations in biopsy size and quality an estimate of 200 fibers were chosen – this was the largest amount of fibers that could be counted if we wanted to include all test subjects. It was then estimated that one image of 882 x 663 microns with aligned muscle fibers holds roughly 100 fibers. Therefore 2 images of 882 x 663 microns were chosen to represent each biopsy. As previously stated, all captured areas were chosen in a blinded manner. Table 5 and table 6 shows the number of fibers counted for each of the two studies involved in this assignment.

Table 5. Fibers counted in Acute study

Time	fibers counted (mean)	SD
Pre	201,2	± 47,5
1 day	206,1	± 53,9
4 day	227	± 63,7
7 day	214,8	± 65,6

Table 6. Fibers counted in Training study

	Fibers counted (mean)	SD
Young		
pre (n = 9)	184,2	± 28,1
post (n = 10)	167,8	± 37,7
Old		
pre (n = 10)	207,9	± 39,5
post (n = 10)	178,6	± 42,8

When all biopsies were analyzed, the method used to determine macrophage content in samples was assessed. The purpose of this assessment was to investigate whether macrophages are evenly distributed over the full biopsy area and whether 2 images are sufficient to estimate the amount of macrophages in a full biopsy sample.

For this investigation, biopsies from the acute study were selected. Biopsies from three test subjects were collected for all time points (PRE, 1D, 4D, 7D) based on biopsy size and biopsy quality. We decided that an appropriate sized biopsy for this evaluation contained at least four separate images (882 x 663 microns) fully covered with muscle fibers.

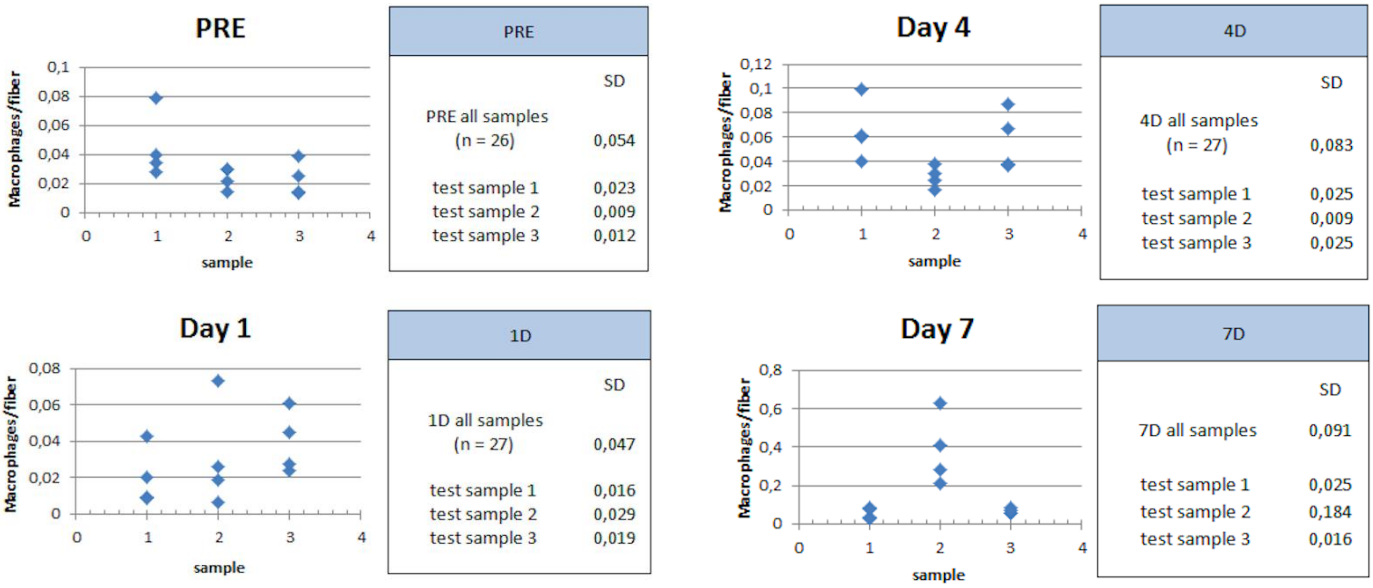

Figure 16. Distribution of macrophages in biopsy samples. Each time point (PRE, Day1, Day4, Day7) is represented by three individual samples, made up by 4 images. Images from the same sample were analyzed and compared using a graphic approach (blue dots). The corresponding table display SD values for each individual sample. Additionally SD values when comparing all subjects are displayed for comparison.

All images/areas from the same muscle sample were analyzed for positive cells (CD68+CD163+ cells/fiber) and compared - this would indicate whether macrophages are evenly distributed in each sample. The findings are presented in figure 16 where results from each time point (PRE, 1D, 4D, 7D) are illustrated graphically. Each blue point in the graph displays the amount of cells counted in one particular image. Additionally, the corresponding tables in figure 16 display SD values based on the four images for each individual sample (sample 1,2,3) and SD values between all subjects in the study for comparison (all samples).

The figure illustrates cell counts from biopsies split into 4 images. As expected, these results revealed that macrophages are not evenly distributed over the entire biopsy area. Some variation appears between each

image in the same biopsy. This variation is defined by a standard deviation (SD) illustrated next to each of the individual test samples in figure 16.

The standard deviation (SD) was generally lower in the individual test samples when compared to the SD value across all samples (Individual samples mean 0.014 vs. SD across all samples PRE: 0.054). This indicates that variation from the individual samples did not markedly influence the results from the total sample size. Increasing the number of analyzed images for each individual biopsy, provides a more precise cell count estimate for that specific biopsy. However, since the variation (SD) across all samples is greater, it appears that the physiological differences between test subjects (inter subject variance) influence the results more than the intravariance from a single biopsy sample. Therefore it is likely that counting additional images (3 or 4 - instead of 2) will only provide minor improvements, whereas adding additional test subjects to the intervention would have a greater impact on results bringing down inter subject variance.

One challenge to the applied method was the low count of macrophages in tissue sections - especially at early time points. In the acute study, mean fibers counted was > 200 to all times. When combining these findings with results from figure 16, absolute cell counts can be estimated, ranging from 4 cells to 8 cells for each person in PRE biopsies. Operating with small observations like these, make data more sensitive to inaccuracies and errors, compared to having large amounts of cells on each image.

3 Results

Data from the acute study is shown first, followed by data from the training study. 2 Additional data graphs, one from each of the two originally intended studies have been included. One illustrates satellite cell content in muscle fibers after an acute bout of exercise and provides useful information for the coupling between satellite cells and macrophages. The other graph offers information regarding myofiber size following 12 weeks of resistance training. This investigation was originally performed by Agergaard (Agergaard et al. 2015), but provide useful information for the interpretations of the results found in this study.

Unless otherwise indicated data is presented as geometric mean ± back transformed SEM. Changes are marked with the asterisk symbol (*) if $P < 0.05$. If $P < 0.001$ changes will be marked with double asterisk (**). Additional symbols may occur, but will be explained below the affected graphs.

Acute Study: CD68+ cells

Figure 17 illustrates changes in muscular CD68+ cell content over time after an acute bout of resistance training. The baseline CD68+ value was 0.037 cells/fiber significantly increased to 0.055 cells/fiber 1day after a bout of heavy resistance training ($P = 0.046$). 4 days after resistance training the CD68+ cells were elevated to 0.075 cells/fiber, significantly higher than pre values ($P < 0.001$). 7 days later CD68+ cells increased even further to 0.104 cells/fiber significantly higher than both pre values ($P < 0.001$) and day 1 values ($P = 0.005$). When analyzing CD68+ cells/mm^2 baseline levels were 6.15 cells/mm^2. CD68+ cells increased significantly 1 day after exercise to 9.11 cells/mm^2 ($p = 0.039$). 4 days after resistance training CD68+ cells were at 12.75 cells/ mm^2, significantly higher than Pre value. Finally, 7 days after resistance training CD68+ cells were at 17.48 cells/ mm^2, significantly higher than pre ($P<0.001$) and day1 values ($P=0.002$).

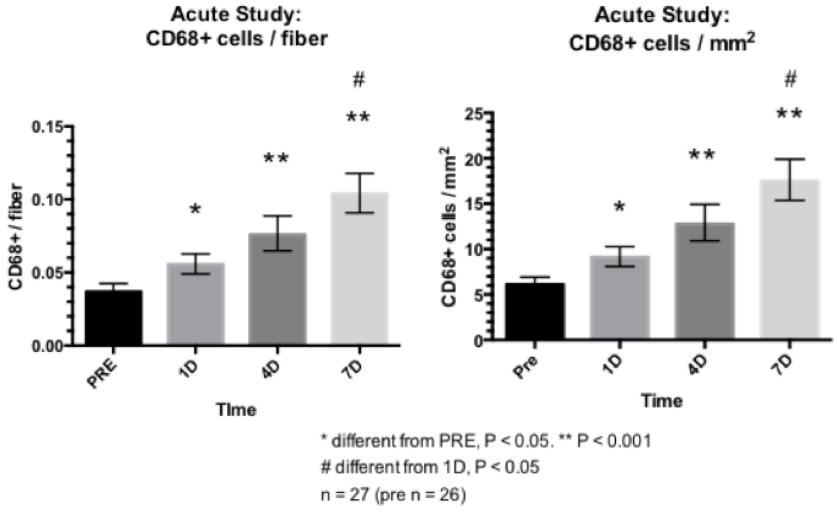

Figure 17. Changes in muscular CD68+ cell content over time following an acute bout of heavy resistance training, (**left**) Illustrated as cells/fiber (**right**) illustrated as cells/mm^2. Values are presented as geometric mean ± back transformed SEM.

3.1 Acute Study: CD68+CD163+ cells

Figure 18 illustrates CD68+CD163+ cells after an acute bout of exercise. No significant changes were seen in CD68+CD163+ cell content 1day after one bout of heavy resistance training: Neither for CD68+CD163+/ fiber or CD68+CD163+/mm^2. 4 days after resistance training CD68+CD163+ cells/fiber were increased to 0.047 cells/fiber. This is significantly higher than pre (0.02 cells/fiber, P = 0.002) and day one (0.029 cells/fiber, P = 0.043). Similarly, CD68+CD163+ cells/mm^2 on day 4 (8.07 cells/mm^2) increased compared to Pre (3.33 cells/mm^2 (P<0.001)) and day 1 (4.86 cells/mm^2 (P= 0.033). On day 7, 0.069 CD68+CD163+ cells/ fiber were found. This is significantly higher than Pre (P<0.001) and day1 (P = 0.002). CD68+CD163+ cells/mm^2 on day 7 were 11.77 . This was higher than Pre (P<0.001) and day 1 values (P=0,001).

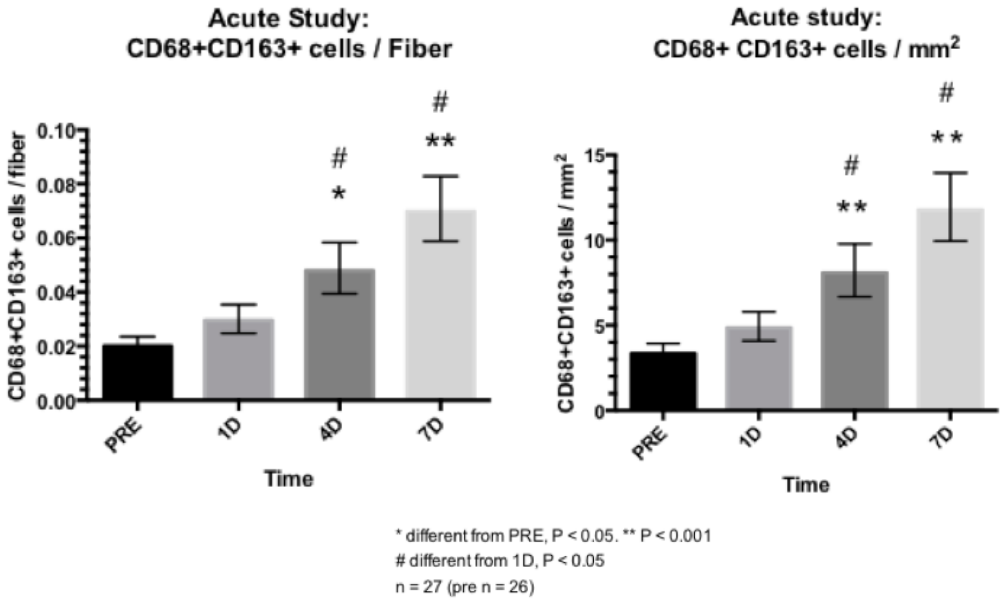

Figure 18. Changes in muscular CD68+CD163+ cell content over time following an acute bout of heavy resistance training, (**left**) Illustrated as cells/fiber (**right**) illustrated as cells/mm^2. Values are presented as geometric mean ± back transformed SEM.

3.2 Acute Study: CD68+CD163- cells

Figure 19 illustrates the content of CD68+CD163- cells following an acute bout of resistance exercise. Baseline value was 0.014 CD68+CD163- cells/fiber. No significant changes were seen on day 1 (0.018 CD68+CD163- cells/fiber). Although not significant, day 4 showed a tendency (0.022 CD68+CD163- cells/fiber, P=0.051) towards an increase after resistance training. 7 days after acute exercise a significant increase in CD68+CD163- cells/fiber is seen (0.026, P = 0.014). When analyzing CD68+CD163- cells/mm^2 no significant changes were seen between baseline (2.308 cells/mm^2) and 1 day (3.021 cells/mm^2). 4day

(3.781 CD68+CD163- cells/mm^2) and 7day (4.398 CD68+CD163- cells/mm^2) both showed a significantly higher number of cells compared to PRE (4day vs. PRE, P = 0,025) (7day vs. PRE, P = 0,005).

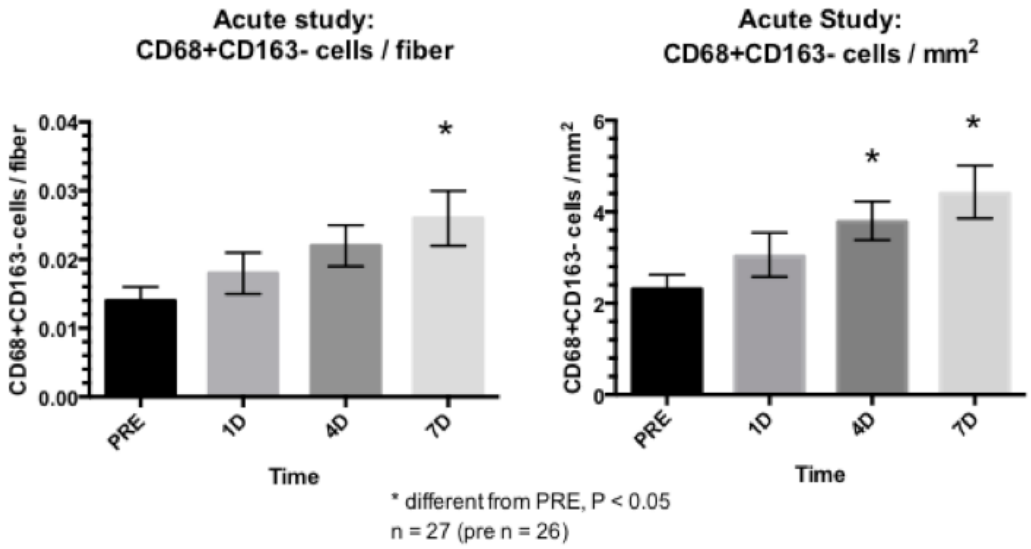

Figure 19. Changes in muscular CD68+CD163- cell content over time following an acute bout of heavy resistance training (**left**) Illustrated as cells/fiber (**right**) illustrated as cells/mm^2. Values are presented as geometric mean ± back transformed SEM

3.3 Acute Study: Satellite cell content

Figure 20 illustrates the Satellite cell content in the Placebo and Losartan group following an acute bout of heavy resistance training. Data in this graph were analyzed in the originally intended study by Heisterberg, (unpublished), explaining why placebo and Losartan data graphically has not been pooled. As previously described in methods, -10d corresponds to the PRE biopsy time point in my investigation. Biopsies to time points -3d and +4,5h has been omitted (from my investigations) and +1d, +4d and +7 corresponds to their original time. There was no significant effect of Losartan on Satellite cell response to exercise, however a main effect of time for SC per type I fiber was found. Mean satellite cell content at baseline in type I fibers was 0,040 SC/fiber for the placebo group and 0,055 SC/fiber for the Losartan group. No changes were seen in satellite cell content at -3d, +4.5h and 1d. 4 days after resistance training, satellite cell content increased to 0,054 for the placebo group and 0,072 for the Losartan group. Since there was no significant effect of drug, losartan and placebo data were pooled. The pooled data were significantly higher at +4d compared to the two groups at baseline (Placebo + Losartan -10d vs. Placebo + Losartan +4d, P < 0,05). Similarly, at +7d Satellite cell count in the placebo group (0,057 SC/fiber) and Losartan group (0,076 SC/fiber) combined was higher than baseline satellite cell content (P < 0.05). No significant changes were seen in type 2 muscle fibers.

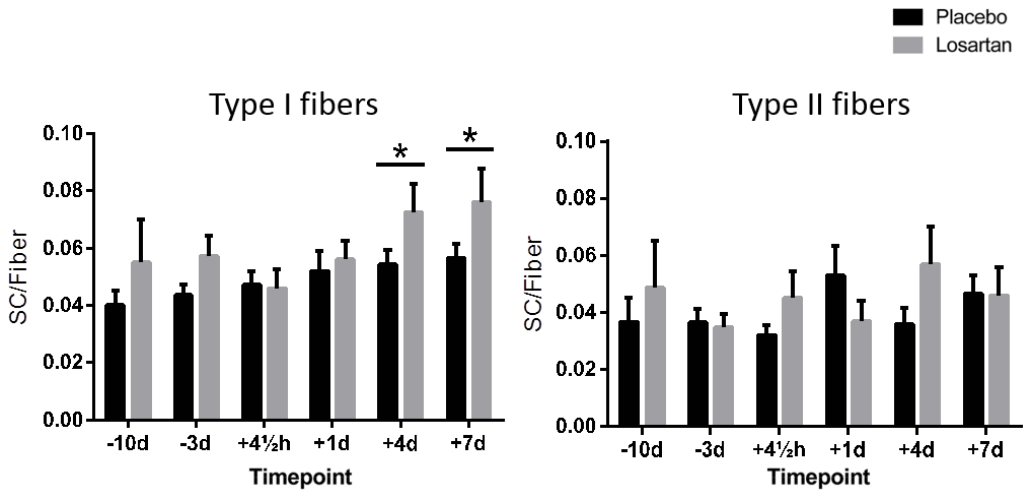

Figure 20. Changes in satellite cell content in the placebo and Losartan group, following an acute bout of unaccustomed resistance training (**left**) in type I fibers (**right**) in type II fibers. Results are presented as mean ± SEM
* different from PRE (P < 0.05)

3.4 Training study: CD68+ cells

Figure 21 illustrates the changes in local CD68+ cell content following 12 weeks of resistance training. No significant changes were seen in CD68+ cell / fiber for the YOUNG group when comparing the PRE(0.053 cells / fiber) and POST (0.042 cells /fiber) group (P = 0.176). In the OLD group CD68+ cell / fiber content increased significantly from 0.047 cells / fiber (PRE) to 0.068 cells / fiber (POST) (P = 0.042). Additionally, when comparing the POST results for the YOUNG and OLD group, OLD had a significantly higher amount of CD68+ cells / fiber (P = 0.014). When analyzing CD68+ cells / mm^2, no effect was seen for TIME. For AGE a significant increase was seen in OLD POST group (10.18 CD68+ cells / mm^2) compared with the YOUNG POST group (6.07 CD68+ cells / mm^2) (P = 0.004).

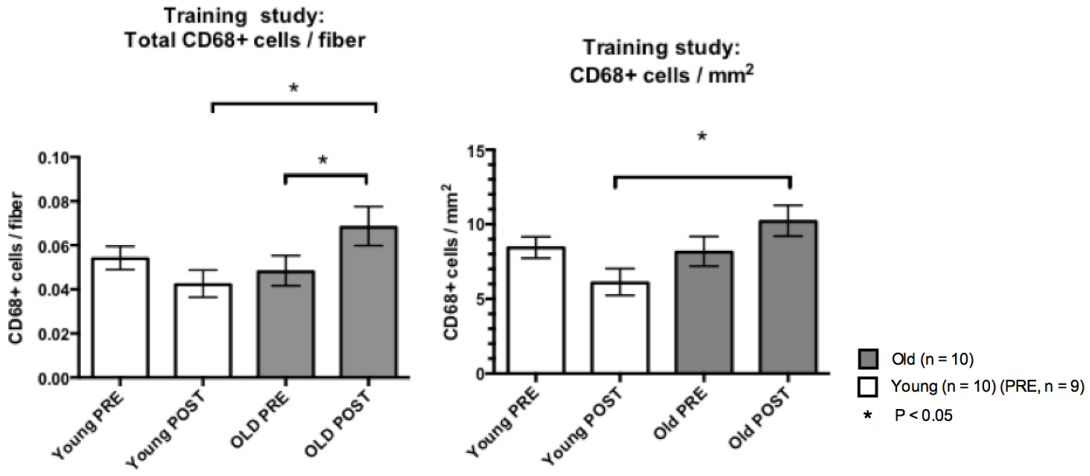

Figure 21. Changes in local CD68+ cell content in young and elderly - before and after a 12 weeks resistance training intervention (**left**) Illustrated as cells/fiber (**right**) illustrated as cells/mm^2. Values are presented as geometric mean ± back transformed SEM.

3.5 Training study: CD68+CD163+ cells

Figure 22 shows changes in CD68+CD163+ cells in the training study. No changes were observed in CD68+CD163+ cells / fiber in the YOUNG group or OLD group following 12 weeks of resistance training. No interaction effect between TIME x AGE was seen for cells / fiber, but an increased number of CD68+CD163+ / fiber in the OLD group compared with the YOUNG group was observed (P = 0.020). CD68+163+ cells / mm^2 results were similar to those just seen. No changes were seen in the YOUNG or OLD group after 12 weeks of resistance training. No interaction effect of TIME x AGE were seen, but a significant difference for AGE illustrated a greater amount of CD68+CD163+ cells / mm^2 in the OLD group compared with the YOUNG (P = 0.006).

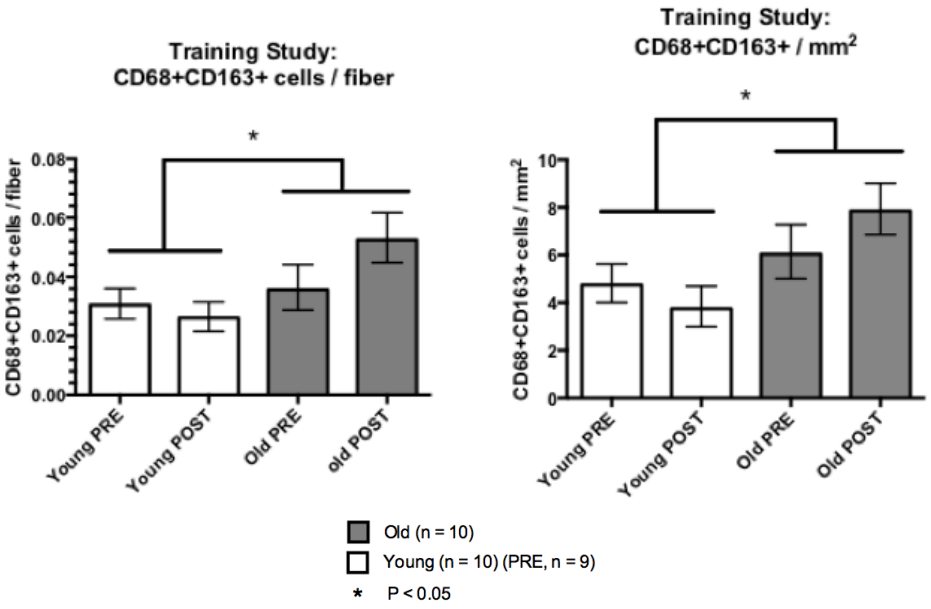

Figure 22. Changes in local CD68+CD163+ cell content in young and elderly - before and after a 12 weeks resistance training intervention (**left**) Illustrated as cells/fiber (**right**) illustrated as cells/mm^2. Values are presented as geometric mean ± back transformed SEM

3.6 Training study: CD68+CD163- cells

Figure 23 illustrates changes in CD68+CD163- cells following 12 weeks resistance training. No time effect was found in the Young or OLD group for CD68+CD163- cells / fiber. A significant interaction effect between AGE x TIME was found (P = 0.019). Within the PRE groups there was a significant difference between the YOUNG group (0.019 cells / fiber) and the OLD group (0.009 cells / fiber) (P = 0.002). No differences were found to the POST time. Results from CD68+CD163- cells / mm^2 also found no effect of time, but found an interaction effect of AGE x TIME. Within the PRE groups there was a significant

difference between the YOUNG (3.10 cells / mm^2) and the OLD group (1.51 cells / mm^2) (P = 0.004). No changes were found within the POST group.

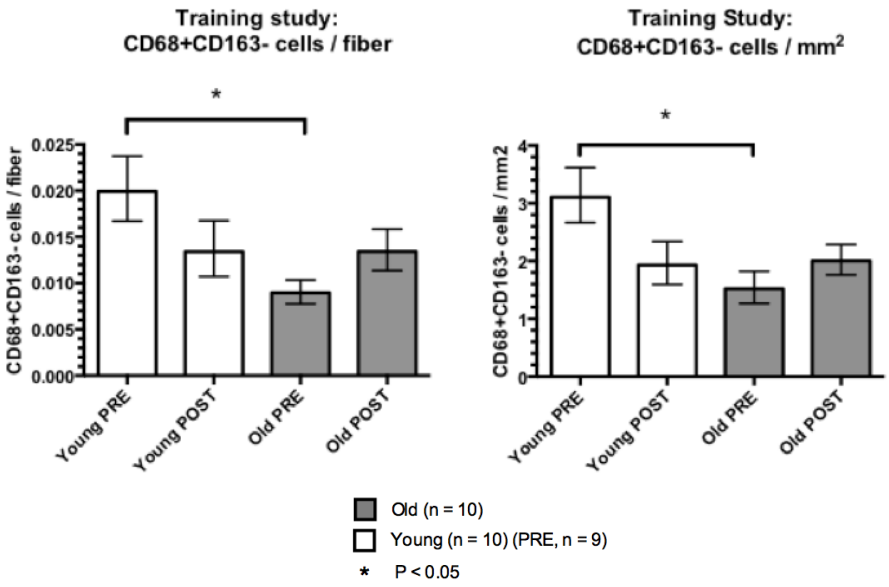

Figure 23. Changes in local CD68+CD163- cell content in young and elderly - before and after a 12 weeks resistance training intervention (**left**) Illustrated as cells/fiber (**right**) illustrated as cells/mm^2. Values are presented as geometric mean ± back transformed SEM

3.7 Training study: Mean fiber area

Figure 24 illustrates changes in mean fiber area for a young and old group of subjects ingesting a vitamin-D or placebo supplement combined with 12 weeks of resistance training. Data in this graph is obtained from Agergaard et al. (Agergaard et al. 2015). For this reason both Placebo and Vitamin D groups are illustrated, although only the placebo group is of relevance for this study. A time effect (PRE vs. POST) was seen in type I and type II mean fiber area for the YOUNG group when combining Placebo and VITD. Young type I fibers at baseline were 4612 μm^2 (VITD) and 4782 μm^2 (Placebo) increasing to 4991 μm^2 (VITD) and 5466 μm^2 (Placebo) after the training intervention. Young type II fibers at baseline were 4663 μm^2 (VITD) and 5202 μm^2 (Placebo) increasing to 5620 μm^2 (VITD) and 6179 μm^2 (Placebo) after the intervention. In the OLD group, no significant changes were seen in type I or type II mean fiber area following 12 weeks of resistance training. Baseline values for the OLD placebo group were 4509 μm^2 (type I) and 4016 μm^2 (type II).

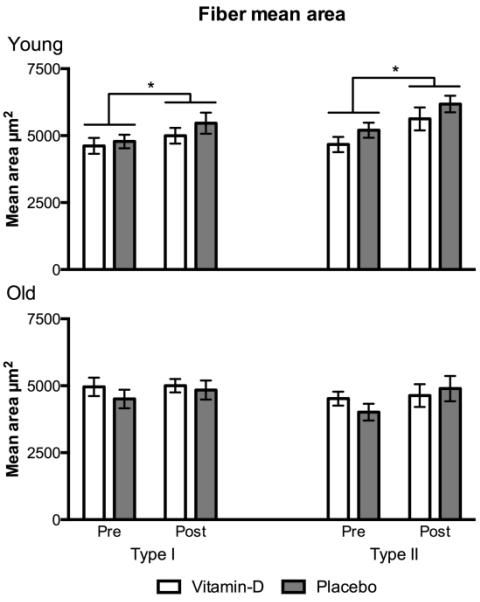

Figure 24. Changes in mean fiber area for type I and type II fibers before and after a 12 week training intervention combined with vitamin D or placebo consumption. (**top**) young subjects (**bottom**) old subjects. Results are presented as mean ± SEM.

4 Discussion

The main focus of this study has been to investigate macrophage kinetics after an acute bout of exercise and after a 12 week training intervention. Results from the acute study indicate that infiltration of macrophages in elderly human muscle occur within 24 hours after a physiological acute damage protocol. During the following days, the presence of macrophages in local tissue gradually increases, reaching the highest observed numbers of CD68+ cells on day 7. Due to limitations of the protocol, this was the last observed time point. When distinguishing between macrophage isoforms in the acute study, increases in anti-inflammatory macrophages were seen 4 days and 7 days after damage. Similar results were seen with infiltration of pro-inflammatory macrophages; however these increases appeared to be smaller.

Following 12 weeks of resistance training, no local changes were seen in macrophage content (CD68+) in young subjects, but an increase in elderly were observed. When analyzing anti-inflammatory macrophages, no effects of training were apparent for subjects in the old or young group, but generally subjects from the old group had increased content of anti-inflammatory macrophages compared to the young group. Pro-inflammatory macrophages were higher in the young group compared to the elderly group prior to the training intervention. After the training intervention though, no changes in inflammatory macrophages were seen when comparing the young and old group.

4.1 Methodological considerations

In order to investigate macrophage infiltration in local muscle, it was necessary to develop a reliable method to analyze macrophage isoforms. CD68+ combined with DAPI was used for detection of macrophage infiltration without distinguishing between isoforms. CD68 has been widely accepted as a marker for macrophage infiltration and has been used in several studies (Przybyla et al. 2006;Saclier et al. 2013; Mackey et al. 2011) and mentioned in several reviews (Tidball and Villalta 2010;Saclier et al. 2013). Additionally, some studies have used CD68 in combination with CD163 or CD206 to identify the anti-inflammatory macrophage isoform (Przybyla et al. 2006;Saclier et al. 2013; Mantovani et al. 2004). Some studies have suggested that CD68 is not a selective marker for inflammation (Paulsen et al. 2012; Gottfried et al. 2008). They have found that CD68 shows reactivity against more cell types than monocytes and macrophages and therefore should be used with great caution when determining inflammatory cells in frozen tissue sections. It is unknown whether these conflicting findings influence the amount of macrophages found in this assignment. However, a majority of cells found in this study have been identified by co-expression of CD68 and CD163. This co-staining strongly suggests that analyzed cells are indeed macrophages. Although some cells have been identified using only CD68, potential errors due to the antibody specificity should have only a minor influence on total macrophage counts, and no influence on anti-inflammatory cell counts.

Pro-inflammatory macrophages are characterized by high levels of INOS and/or TNF-α expression (Saclier et al. 2013; Greiwe et al. 2001; Mosser and Edwards 2008;Chazaud 2015), but only a limited number of studies have used iNOS or TNF-α antibodies for the detection of this macrophage isoforms. As described earlier under methodological consideration, we managed to come up with a staining protocol to identify macrophages (using CD68+DAPI) and anti-inflammatory macrophages (using CD68+CD163+DAPI) in human muscle tissue, however INOS and TNF-α did not seem to be reliable markers for identification of pro-inflammatory macrophages in this study. Staining with these antibodies was characterized by an overexpression of the markers in tissue sections. Various tissue staining protocols were tested for identification of pro-inflammatory macrophages including regulation of antibody concentration and background blocking, but no effective method was found using iNOS or TNF-α. An immunohistochemistry protocol usually involves a set of procedures necessary for successful staining. These procedures are often extremely sensitive to changes in concentration and timing. Therefore we cannot rule out, that factors other than the primary antibodies (iNOS/TNF-α) could have played a role for the results seen in this study. It is possible that iNOS or TNF-α could function as reliable markers for pro-inflammatory macrophages in human tissue under different settings or other protocols, however none of the tested protocols used in this experiment were successful.

We defined macrophages that were not anti-inflammatory (CD68+CD163+) as pro-inflammatory. This method, however, does not provide an accurate picture of infiltration, and should be considered only as a rough estimate of events, due to a lack of better options. Although the quality of the fluorescent CD163 and CD68 staining was generally high, making it easy to distinguish cells expressing CD163 and CD68 from those only expressing CD68, this method was occasionally challenged by cells partially expressing CD163 making their classification as pro or anti- inflammatory difficult.

To avoid areas of perimysium, two nonoverlapping images of 882 x 663 µm were analyzed from each biopsy. In untrained elderly subjects, as those used in the acute study, each image was estimated to contain 100 fibers, resulting in a mean of > 200 fibers being analyzed for each person. Our 12 week training study involved old subjects, young subjects and a training regime. Resistance training and age are factors that influence myofiber size (Lexell, Taylor, and Sjöström 1988; Andersen 2003). This is evident when analyzing myofiber count data from this present study. Fewer fibers were counted after the training intervention in young and old subjects, which may imply increases in fiber size. If muscle fiber size increases, fewer fibers will fit into the microscope image (Table 6). In order to compare young and old subjects before and after a training regime,

Table 6. Fibers counted in Training study

	Fibers counted (mean)	SD
Young		
pre (n = 9)	184,2	± 28,1
post (n = 10)	167,8	± 37,7
Old		
pre (n = 10)	207,9	± 39,5
post (n = 10)	178,6	± 42,8

it is necessary to standardize to cells/fiber in addition to cells/mm^2. Results based simply on cells/mm^2 are not sufficient to explain actual observations, because increases in fiber size alter the image composition – resulting in more fiber area and less connective tissue. Therefore, when analyzing results, information about cells/fiber should be available for a better estimate of events.

As we have previously seen, some complications are associated with selection of 2 images. It appears that macrophages are unevenly distributed throughout the biopsy. The result from the image analysis may therefore differ within the same biopsy depending on the selected area. To avoid bias, area selection was chosen using DAPI images. Additional criteria for images were fibers covering the entire image (height and width). Biopsy cross sections vary greatly in quality and size. The protocol used in this investigation was intended to include the largest possible number of muscle fibers, without excluding a large number of subjects due to limitations in biopsy size. Another possible method could have been to analyze macrophage content in the entire biopsy. This method is sometimes applied when analyzing satellite cells; however this method would involve other difficulties such as avoiding/subtracting the perimysium where large numbers of infiltrating macrophages reside. A limited number of studies have investigated pro- and anti-inflammatory macrophages in human tissue, which leads to limited number of tested protocols for macrophage detection. One study by Przybyla (Przybyla et al. 2006) investigated macrophages using at least two fields corresponding to 637 µm x 475 µm. The method by Przybyla is similar to the one used in this present assignment, however this present study uses larger images to represent the sample biopsies. As discussed under methodological considerations one problem when counting macrophages in muscle sections is the low presence of cells per fiber. When operating with small observations, data are more sensitive to inaccuracies and errors. Therefore the general aim should be to analyze the highest possible number of fibers in all biopsies.

4.2 Local CD68+ cells following acute unaccustomed bout of heavy resistance training

The purpose of the acute study was to investigate changes in muscle macrophage content and response following a physiological bout of unaccustomed exercise. Findings from this study reported that changes in local macrophage content (CD68+) were seen 24 hours after the damage protocol. CD68+ cell content is expected to increase following a damage protocol, however an increase in CD68+ cells 24 hours post injury is surprisingly fast considering the findings from previous studies. A study by Przybyla (Przybyla et al. 2006) found no changes in CD68+ cells 3 days after an acute bout of exercise in elderly, whereas a study by Paulsen (Paulsen et al. 2012) found the highest CD68+ cell counts on day 4 and day 7 after eccentric exercise of the elbow flexors. A review by Tidball (Tidball and Villalta 2010) suggests that invading phagocytic macrophages reach significantly elevated concentrations 24 hours post injury, but these findings were based on animal studies questioning their application to humans.

This study found that the number of CD68+ cells gradually increase the following days reaching a peak 7 days after the heavy bout of unaccustomed resistance training. These results are similar to results found by Paulsen (Paulsen et al. 2012) under physiological circumstances, Saclier (Saclier et al. 2013) using electric stimulation and Tidball in animal studies (Tidball and Villalta 2010). Due to limitations, this study cannot examine whether the amount of CD68+ cells continuously increase after 7 days, reaching even higher numbers. Also the duration for the increased number of CD68+ cells remains unknown, a study by Mackey (Mackey et al. 2011) has reported increased numbers of CD68+ cells 30 days after a damage protocol. However, damage in this study was induced using electric stimulation and may therefore not imitate scenarios caused by physiological work.

4.3 Local changes in macrophage subtypes following acute bout of resistance training

When distinguishing between pro- and anti-inflammatory macrophages PRE values for anti-inflammatory macrophages seemed slightly higher than pro-inflammatory (Pro: 0.014 / fiber, Anti: 0.020 /fiber), however following the bout of heavy exercise increases in anti-inflammatory macrophages seems to be more pronounced than increases in pro-inflammatory macrophages, increasing 245% and 85%, respectively. These findings are similar to results from a study by Przybyla. Although their analysis generally found a higher absolute number of macrophage to the different time points, they found that a majority of macrophages in skeletal muscle display anti-inflammatory characteristics and that a relatively small subpopulation of macrophages dictates the pro-inflammatory response following an acute bout of exercise. Based on these findings it seems that anti-inflammatory macrophages constitute a larger quantity of macrophages present in the muscle than pro-inflammatory macrophages.

According to literature, local inflammation is characterized by a pro-inflammatory response occuring prior to an anti-inflammatory response. Particularly, this has been shown in studies where damage has induced cell necrosis and regeneration of muscle fibers. Studies have found that interfering with the sequenced polarization of macrophages strongly alters muscle regeneration (Chazaud 2014). In this present study an increased amount of local anti-inflammatory macrophages (CD163+) were seen on day 4 and day 7 post injury in cells/fiber and cells/mm^2. Compared to baseline, local pro-inflammatory macrophages (CD163-) were significantly higher on day 4 and day 7 for cells/mm^2, however, when analyzing fiber/mm^2 significant increases were seen on day 7, but not for day 4 ($p = 0.051$). These results suggest that pro- and anti-inflammatory infiltration occur almost simultaneously. This is in contrast to the previously described local infiltration pattern, suggesting an increased number of pro-inflammatory macrophages prior to the anti-inflammatory macrophage response. The findings in this assignment could be explained by limitations of the chosen protocol. This study only investigates macrophage content at certain time points (PRE, 1D, 4D, 7D)

however it is likely that an increase in pro-inflammatory macrophages could be observed on day 2 or day 3, prior to the anti-inflammatory macrophages detected at day 4. Another explanation for simultaneous infiltration of macrophage isoforms could relate to the findings in a study by Saclier (Saclier et al. 2013). This study found that macrophage isoforms coexisted in damaged tissue. Fibers in the same area were classified as either early regenerating fibers or late regenerating fibers using myogenin. Early regenerating fibers express increased content of pro-inflammatory macrophages, whereas late regenerating fibers expressed increased content of anti-inflammatory macrophages. These findings suggested that pro- and anti-inflammatory macrophage infiltration possibly overlap spatially and temporally in local tissue, in contrast to being two separate events. Such findings could support the results obtained from this acute study, where pro- and anti-inflammatory macrophages seems to reside in the local tissue simultaneously. The training protocol selected for this experiment clearly plays a crucial role for the missing damage response. One purpose of this study was to investigate changes in local macrophage content following a physiological bout of exercise. As previously described, current knowledge on local macrophage content is mainly based on findings from studies with muscle damage and regenerating fibers. Muscle regeneration is defined only as a response to situations where myonecrosis has first occurred (Grounds 2014), difficult to induce under physiological setings. In such regeneration studies, pro-inflammatory macrophages are believed to perform phagocytosis clearing cell debris from damaged fibers (Bénédicte Chazaud et al. 2009). In this study, no regenerating fibers were observed for any subject. This emphasizes the theory that myofiber necrosis is almost impossible to induce under physiological settings. Additionally, a faded or missing early pro-inflammatory response as seen in my results could be linked to the absence of damaged myofibers. Though speculative, it is possible that since no dead fibers were present in local tissue, no urgent pro-inflammatory response is necessary to clear out cell debris, resulting in a diminished or delayed pro-inflammatory response.

4.4 Macrophages and satellite cells

Cytokines excreted from the muscle in response to inflammation and exercise has been hypothesized to affect systemic inflammation profile (Petersen and Pedersen 2005). Additionally, studies have shown that cytokines expressed by macrophages influence stages of satellite cell proliferation, differentiation and fusion (Arnold et al. 2007). Data on satellite cell content in the acute study was included from the original research paper by Heisterberg (unpublished). These results found an increased satellite cell content 4 days and 7 days after a bout of heavy resistance training in type I fibers. A similar study by Nederveen (Nederveen et al. 2015) found increases in SC content only in type I fibers, however these increases were seen 24h and 48h following resistance exercise. Snijders (Snijders et al. 2015) argues that satellite cell content following an acute bout of exercise increases in the following few days and peaks 72 hours post exercise, but does not go into fiber type specific considerations (Snijders et al. 2015).

When comparing satellite cell data from the acute study with findings from this study, it seems that local macrophages and satellite cell content increase at a similar time. CD68+ cells were upregulated 24 h post injuries, steadily increasing until the last measured sample at day 7. When distinguishing between subtypes, increases in CD68+CD163+ (anti) cells and CD68+CD163- (pro) were seen on day 4 and day 7. This study setup does not allow for any detailed investigations of the relationship between macrophages and satellite cells, the findings, however, indicate that macrophages and satellite cells are upregulated in local muscle simultaneously. It is possible that regulation of satellite cells is administered by cytokines from macrophages. Especially pro-inflammatory macrophages have been suggested to contribute to the proliferation and growth of muscle precursor cells (Arnold et al. 2007). The time frame for upregulation of macrophages and satellite cells elucidated in this study supports the theory provided by Arnold, suggesting a close interaction between satellite cells and macrophages. Also, this present study found that satellite cells increased in type I muscle fibers, but not in type II fibers. This was supported by findings in other studies. Activated satellite cells are associated with muscle stretch, trauma or muscle overload. The findings from this current study could indicate that greater stress have affected type I fibers. This is likely to occur, as type I fibers are generally associated with an early recruitment compared to type II fibers during muscle loading. Type I fibers may therefore suffer more stress/damage compared to type II muscle fibers, expressed by additional satellite cells around these fibers. We did not investigate macrophages in relation to fiber type; however it would be interesting to investigate whether increased macrophage content would appear at sites where satellite cells were upregulated following muscle damage, in this case around type I muscle fibers.

4.5 Mean fiber area following a 12 weeks training intervention

The acute study illustrated a clear anti-inflammatory response 7 days following an acute bout of resistance training. It was then hypothesized that regular resistance training would provide or maintain a heightened anti-inflammatory environment in the muscle, expressed by an increased number of anti-inflammatory macrophages. The purpose of the training study was therefore to examine changes in local tissue following 12 weeks resistance training. Data on mean fiber area was included from the original study by Agergaard (Agergaard et al. 2015). As expected, 12 weeks of resistance training with 3 sessions/week increased fiber mean area of type I and type II fibers in the young group. No effect was seen in the elderly group before and after training for type I or type II fibers. Previous studies have found increases in mean fiber area in elderly following a 12 week resistance training intervention – especially in type II fibers. A study by Verdijk tested 13 men elderly men (aged 72) and observed significant increases only in type II mean fiber area (Verdijk et al. 2009). Another study by Esmarck found similar results in elderly men aged 74. This study reported increases in type II mean fiber area, but no changes in type I mean fiber area (Esmarck et al. 2001). It is therefore surprising that no changes were seen in the present study by Agergaard. According to the study

there was a tendency towards a time effect of type II fibers (p = 0.074), Agergaard therefore speculates that the training period in the study was too short for the elderly to adapt to the chosen resistance training protocol.

4.6 CD68+ cells in resting muscle

The first finding from the 12 week training study is that the number of macrophages in skeletal muscle is low when no prior damage has been induced to fibers. Data from this training study found 0.053 CD68+ cells/fiber in young muscle and 0.047 CD68+ cells/fiber in old muscle. Baseline values in the acute study (from this assignment) found that elderly had 0.037 CD68+ cells/fiber. In both experiments (acute study and 12 week training study) some muscle cross sections showed no or very little CD68+ activity when analyzed, indicating that no macrophages were present in the tissue at that time. These findings are consistent with findings from another study investigating macrophage content in young and elderly by Charmaine (Charmaine and Sparks 2012). Charmaine reported CD68+ content as percent muscle macrophages (macrophages/fibers x100) and found that macrophage content in a resting state was 4.7 % in young muscle and 4 % in old muscle. When presenting numbers from this current assignment in a similar way macrophage content is 5.3 % in young muscle and 4.7 % in old muscle. Interestingly, the study by Charmaine found that CD68+ cells were only present in 12 (6 young, 6 old) out of 25 skeletal muscle samples. Although large interpersonal variations occur, these findings suggest that macrophage content in the rested muscle is limited; sometimes macrophages even seem to be absent. The presence of macrophages in resting tissue could be speculated to have some important regulatory properties, maintaining an optimal environment for neighboring cells. Another important role for resident macrophages could be to quickly respond to foreign substances, functioning as a first line of defense. Resident macrophages have been theorized to produce cytokines that are important for recruitment of neutrophils and monocytes to the inflammation site. However since resident macrophage content regardless of age and training appear to be minimal, it could be speculated that low quantities of resident macrophages are sufficient for recruitment of immune cells during infection/damage or that other local cells contribute to the recruitment as well. Macrophages (CD68+) in resting muscle were also investigated in a study by Mackey (Mackey et al. 2011). Most of her observations report macrophage content lower than 15 cells/mm^2 in resting muscle, which is similar to findings in this study where 8.4 cells/mm^2 and 8.1 cells/mm^2 were found for young and old resting muscle, respectively. Additionally, some observations from the study by Mackey ranged around 1-2 cells/mm^2, supporting the theory that in some muscle samples, macrophages are hardly detected. These findings taken together indicate that CD68+ observations across studies are similar. Despite great individual variations between subjects these presented studies have observed similar macrophage content expressed as cells/fiber or cells/mm^2. A study by Przybyla reported much higher CD68+ observations before and after a training intervention than those seen in other studies (Przybyla et al. 2006). With no prior muscle damage, macrophage content is local

tissue is generally low. This presents some difficulties when analyzing data from biopsies with no prior damage.

4.7 CD68+ following a 12 weeks resistance training intervention

In this study the old group had significantly higher content of CD68+ cells/ mm^2 when compared to the young group after a training intervention. As previously described cell counts illustrated pr. mm^2 do not necessarily express actual circumstances. Old muscle fibers are generally smaller than young muscle fibers, which influences the proportions of the image. Images are composed of muscle fibers and connective tissue. Muscle fibers will take up a more area relative to connective tissue in images with big fibers, which may influence cell counts, as macrophages are located in the connective tissue. With regards to that, data has been illustrated as cells/ fiber in addition to cells/mm^2.

When analyzing CD68+ cells/fiber significant increases in CD68+ cells were observed following 12 weeks of resistance training for the old group, but not for the young group. Additionally, no TIME effect were observed for anti- (CD68+CD163+ cells) or pro-inflammatory macrophages (CD68+CD163- cells) for any of the groups. This indicates that macrophages do not seem to accumulate in tissue during a training intervention. Despite increases in CD68+ cells in the OLD group following 12 weeks of resistance training, no accumulative effects were observed for any macrophage isoform. To our knowledge no previous studies have tested the hypothesis of macrophage accumulation in healthy elderly following a training intervention. We hypothesized that resistance training would cause upregulation in local macrophage content in the resting muscle. The assumptions were that increased quantities of anti-inflammatory macrophages in resting muscle following a training intervention, could protect the muscle against symptoms associated with chronic increases in pro-inflammatory cytokines. Though speculative, it was hypothesized that an increased anti-inflammatory local environment could even affect the systemic circulation and influence low grade inflammation, although additional investigations are necessary to test this thesis. The findings from this study though suggest that no such accumulation of macrophages seems to appear, since no additive effects of macrophages were present for any macrophage isoform.

It is possible that the findings in this assignment, reporting increased content of CD68+ cells in the OLD group does not reflect the entire training intervention, but simply a delayed damage response to one individual training session prior to the biopsy extraction. This could explain the elevated number of CD68+ cells in the elderly group. This, however, seems unlikely due to the intensity of the exercise protocol and the repeated bout effect. The *repeated bout effect* covers the adaptation of tissue to a repeated stimulus. This means that muscle damage may appear when subjects are unaccustomed to an exercise. However, when the same exercise is performed again within a certain period of time, it does not induce as severe muscle damage

as that induced previously (Mchugh 2003). Since the elderly in this study have had several weeks to adapt to the training intervention, this explanation for the results seems unlikely.

Though speculative, another explanation for the differences observed in CD68+ cells in the old group following resistance training, could be that the response to heavy unaccustomed exercise in elderly is delayed compared to the young group. Mackey illustrated that macrophage content (CD68+) was heightened 30 days following electric stimulation in young subjects, suggesting that muscle adaptation to damage is an extensive process of long duration (Mackey et al. 2011). Damage was not induced in this study, but the young group may have adapted to the training intervention faster than the elderly group. The increases in elderly CD68+ may therefore simply express a prolonged resolution of inflammation in elderly.

4.8 Changes in macrophage subtypes following a 12 week resistance training intervention

We hypothesized that young muscle contained a more anti-inflammatory environment compared to muscle in elderly expressed by an increased number of anti-inflammatory macrophages. Additionally, we expected that a 12 week resistance training intervention would alter local inflammatory profile contributing to a less inflamed muscle environment. In literature macrophage subpopulations have not been investigated extensively, therefore previous findings are scarce. A study by Lee investigated macrophage isoforms and their correlation to strength gains in elderly. This study found that 12 weeks of resistance training in elderly significantly increased anti-inflammatory macrophages, whereas no changes were seen in pro-inflammatory macrophages (Lee and Peterson 2012). Endurance exercise has been related to elevated anti-inflammatory release in systemic circulation and increases in anti-inflammatory macrophages have been observed following one bout of unaccustomed heavy resistance training. The main finding from this 12 week resistance training intervention was that no effect of time were seen for pro (CD68+CD163+) or anti-inflammatory macrophages (CD68+CD163-). Based on these findings, resistance training does not seem to alter/accumulate local macrophage content significantly.

Another surprising finding was that elderly in general seemed to have a higher content of anti-inflammatory macrophages compared to the young group. One possible explaining for this could be the link between anti-inflammatory macrophages and connective tissue. As previously described, anti-inflammatory macrophages secrete factors that dampen inflammation and stimulate growth. Transforming growth factor-β (TGF- β) is one of such factors, characterized by pro-fibrotic functions. It is speculated that an increased and persistent presence of macrophages modifies the intensity and duration of released growth factors from especially anti-inflammatory macrophages. This consequently could lead to excessive extracellular matrix (ECM) accumulation and replacement of muscle with fibrotic tissue (Mann et al. 2011; Lech and Anders 2013). Aging is associated with a loss in muscle mass and accompanying increases in fibrotic tissue (Mann et al. 2011). It is possible that the increased content of anti-inflammatory macrophages found in this present study

could therefore be related to structural changes in connective tissue rather than an inflammatory response in the muscle. A study by Novak (Novak, Weinheimer-Haus, and Koh 2014) found that increased pro-inflammatory macrophage content in muscle tissue decreased fibrosis and enhanced fiber regeneration in lacerated muscle. These findings could be a possible explanation for the increased content of pro-inflammatory macrophages seen in the young muscle compared to muscle from elderly. An increased content of pro-inflammatory macrophages may actually benefit muscle regeneration, and the findings that pro-inflammatory macrophages are lowered in muscle from elderly could again suggest the possibility that macrophage counts as those conducted in this study are related to structural changes in the ECM rather than inflammation.

4.9 General considerations

When investigating inflammation several difficulties arise. The immune system is composed of a large number biological structures and processes within the organism. The immune system is a host defense system that protects the body from foreign objects and diseases. The primary focus of investigation in this assignment has been macrophages; however we cannot rule out that other immune cells are equally important for regulation of inflammation. T-lymphocytes, B-lymphocytes and mast cells, also secrete cytokines – some which are similar to those expressed by macrophages (Mosser and Edwards 2008). Due to the complexity of the immune system and the interaction between various cells types, simply investigating macrophages may not provide the full picture of inflammation. Another major complication when analyzing macrophages and inflammation is that we do not know what constitutes an appropriate response to muscle damage or training in general. One purpose of this study has been to illustrate the inflammation process following an acute bout of unaccustomed resistance training in elderly. Despite some insight through animal studies, it remains to be elucidated whether a prolonged response to damage in humans is beneficial or unfavorable - and how a response in elderly corresponds to young subjects. Such future studies could be helpful for a better understanding of inflammation and the immune system. Other future findings could investigate the connection between local and systemic inflammation. As we have seen, some studies have proposed a myokine effect on chronic inflammation (Petersen and Pedersen 2005), but the connection between local- and systemic inflammatory factors still remains unclear.

From a methodological point of view it could have been interesting to combine findings from immunohistochemistry with gene expression analysis of different cytokines related to macrophage isoforms. This could support our findings and indicate potential changes in macrophage activity following our interventions.

5 Conclusion

Studies have shown that local inflammation in response to muscle damage is tightly orchestrated by macrophages, playing an important role for successful muscle regeneration and muscle maintenance. Additionally, aging has been associated with chronic levels of heightened inflammation, which has been linked to loss of muscle strength and mass in elderly. Studies on local inflammation following resistance training in humans are sparse, possibly due to the current available methods for detection of macrophages.

In the present study we were able to develop a reliable method for detection of anti-inflammatory macrophages, but TNF-α and iNOS were not successful for detection of pro-inflammatory macrophages. Instead we attempted to distinguish pro- from anti-inflammatory macrophages using CD68+CD163- (pro) and CD68+CD163+ (anti). With this protocol we observed that macrophage infiltration (CD68+) occurs early after a heavy bout of resistance training (24 hours), and gradually increases over the next 7 days post exercise. Pro- and anti-inflammatory macrophages increased simultaneously on day 4 and day 7 and this coincided with increased satellite cell content in type I muscle fibers.

Following a 12 week resistance training intervention, no changes were detected in resting muscle for pro- or anti-inflammatory macrophage isoforms. These findings indicate that macrophages do not accumulate following a resistance training intervention. Another interesting observation in the elderly and young groups was that macrophage content in skeletal muscle was very low or absent when no prior damage had been induced to fibers.

Contrary to previous beliefs, muscle in elderly contained more anti-inflammatory macrophages than young muscle. This could be related to macrophage functions in situations other than simply inflammation, highlighting the complex nature of macrophages.

In conclusion, this study finds macrophage response to acute training induced muscle injury, but no adaptation following a resistance training intervention. The findings from this study are not sufficient to explain the function of macrophages and their connection to Sarcopenia. Future studies should aim to locate a reliable antibody for detection of pro-inflammatory macrophages, whereas other studies should investigate the interaction between local and systemic inflammation.

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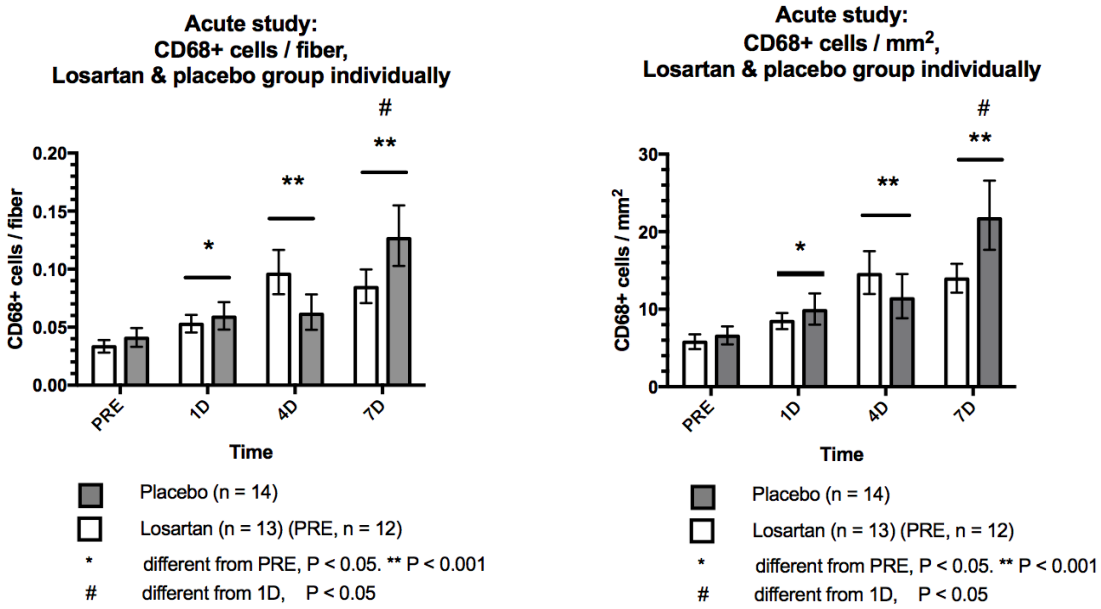
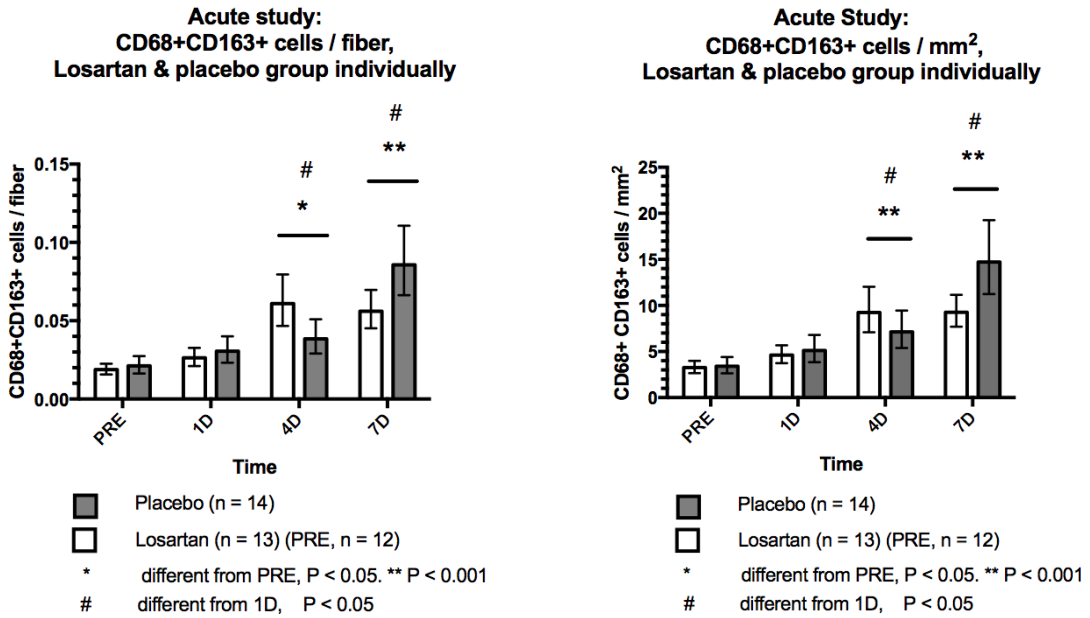
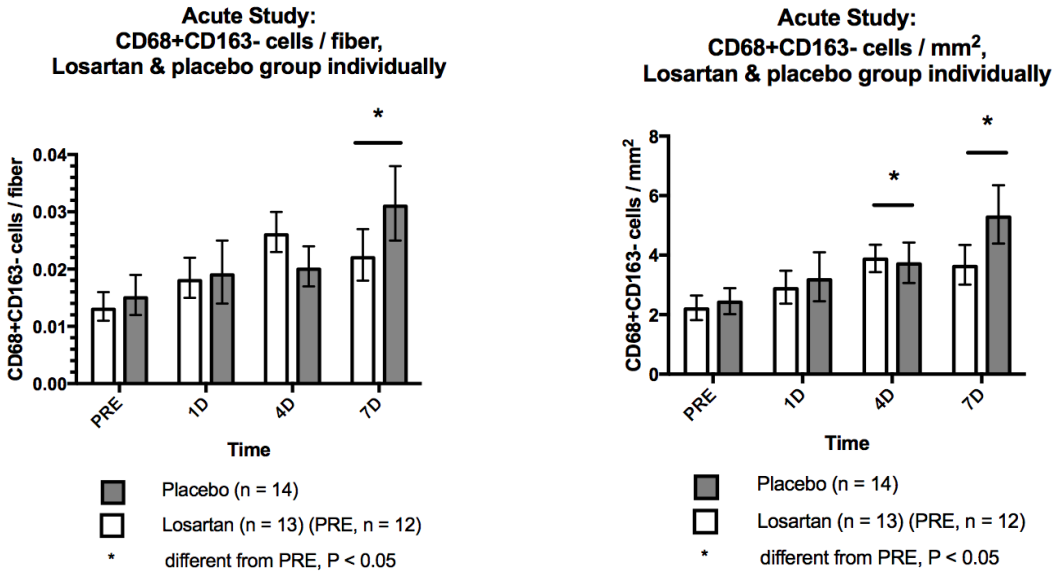
Appendix



Appendix 1. Figure A. Changes in muscular CD68+ content following an acute bout of heavy resistance training. Original data for the Placebo and Losartan group individually (**Left**) presented as cells / fiber (**Right**) presented as cells / mm^2 These groups were pooled in the assignment, since no effect of Losartan consumption was found. Data is presented as geometric mean ± back transformed SEM.



Appendix 1. Figure B. Changes in muscular CD68+CD163+ content following an acute bout of heavy resistance training. Original data for the Placebo and Losartan group individually (**Left**) presented as cells / fiber (**Right**) presented as cells / mm^2 These groups were pooled in the assignment, since no effect of Losartan consumption was found. Data is presented as geometric mean ± back transformed SEM.



Appendix 1. Figure C. Changes in muscular CD68+CD163- content following an acute bout of heavy resistance training. Original data for the Placebo and Losartan group individually (**Left**) presented as cells / fiber (**Right**) presented as cells / mm^2 These groups were pooled in the assignment, since no effect of Losartan consumption was found. Data is presented as geometric mean ± back transformed SEM.