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System-level analyses to identify macrophage-specific mechanisms controlling skeletal muscle regeneration

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The Examination took place at Department of Molecular Biology and Biochemistry, Faculty of Medicine, University of Debrecen at 11 a.m., 10th of July 2017

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Introduction

1. Inflammation

- **Overview**

The four principal signs of inflammation are known as "Celsus tetrad": calor (heat), dolor (pain), tumor (swelling) and rubor (redness). However, the past decades offered greater insights on how inflammation is initiated, sustained and resolved. Inflammation is a physiological protective response, initiated after infection or injury. Inflammatory responses taking place either locally or systemically aim to eliminate threats, and to promote tissue repair and healing. In the case of infection, immune memory needs to be established in order for the host to mount a faster response upon secondary encounters. Thus, in general a controlled inflammatory response is beneficial against infection, but it can become harmful, if dysregulated. Many types of inflammation-related responses are only known in pathological conditions but the physiological initiation counterpart, which is an important piece of the puzzle, is not known. Since inflammation seemingly evolved as a response for restoring homeostasis, elucidating the mechanisms that drive physiological inflammation will help to understand the dysregulated inflammatory processes that lead to systemic chronic inflammatory states.

Through evolution vertebrates have developed a complicated recognition system in order to effectively guard themselves, which involves both fast acting innate and slowly activated adaptive immune responses by delivering plasma and leukocytes to the site of infection or injury. Many cell types contribute to these immune responses but in this dissertation, we will focus on the different types of macrophages and will try to dissect their roles. Innate immune cells such as monocytes and macrophages are able to detect specific molecules through their pattern recognition receptors (PRRs) and thus trigger the initiation events of inflammation. This infection recognition response has been characterized best for bacterial infections through specialized receptors called Toll-like receptors (TLRs) and NOD (nucleotide-binding oligomerization-domain protein)-like receptors (NLRs). Tissue-resident macrophages and mast cells, mediate the initiation of the inflammatory response by producing inflammatory mediators such as chemokines, cytokines, vasoactive amines, eicosanoids and products of proteolytic cascades. These mediators help plasma proteins and leukocytes (mainly neutrophils) to enter the tissues at the site of infection (or injury) while at the same time prevent the exit of erythrocytes. Neutrophils become activated either by direct contact with pathogens or through the actions of cytokines secreted by tissue-resident cells, and kill the invading pathogens by releasing the contents of their granules (reactive oxygen species, reactive nitrogen species, proteinase, cathepsin and elastase). However, the actions of neutrophils do not discriminate between pathogen and host targets. Whereas sustained inflammation is essential to battling pathogens, excessive inflammation can lead to severe tissue damage. Therefore, immune responses, physiologically, are tightly regulated and in many cases self-restricted via negative feedback signaling loops. For example, the switch in lipid mediators (from prostaglandins to lipoxins) is critical for the resolution of inflammation. Resolvins, protectins, transforming growth factor-β and growth factors, produced by infiltrating macrophages, also have a crucial role in the resolution of inflammation and the initiation of tissue repair.

As mentioned above a large number of mediators are produced during the inflammatory response. For simplicity, they are classified as inducers, which act as signals to initiate the inflammatory response, and mediators that act as effectors of inflammation. Inducers can activate specialized sensors, which in turn lead to the production of specific sets of mediators. Thus, inflammation consists of inducers, sensors, mediators and effectors, expressed and produced mainly by macrophages, thus defining the type of inflammatory response. In the following sections, these components are discussed.

- **Classical and alternative polarization of macrophages**

Macrophages are cells that are characterized by extended flexibility and plasticity. Under various signals such microbial products or damaged cells macrophage undergo either classical M1 activation or alternative M2 activation. M1 polarized macrophages *in vitro* are activated by various TLR ligands and IFN-g, while M2 polarized macrophages are activated by Interleukin-4 and Interleukin-13 (IL-4/IL-13). In general, M1 macrophages express high levels of pro-inflammatory cytokines and reactive nitrogen intermediates (iNOS) and thus act strongly against microbes. On the other hand, the M2 phenotype is characterized by anti-parasitic activity, tissue remodeling, wound healing and tumor sustained progression through its immunoregulatory activity (Tumor Associated Macrophages). In addition, M2 macrophages have high expression of scavenging molecules, produce L-ornithine through the arginase pathway and are distinguished by low levels of IL-12 and high levels of IL-10. Furthermore, M1 versus M2 macrophages have different chemokine expression profiles.

Transcription factors, posttranscriptional regulators, sensors and signaling molecules are involved in regulating macrophage activity. For example, the balance between activation of Signal transducer and activator of transcription-1 (STAT1) and STAT3/STAT6 fine-tunes macrophage activity and subsequently polarization. In this context, NF-κB and STAT1 activation promotes M1 macrophage polarization, while STAT3 and STAT6 activation results in M2 macrophage polarization, which is associated with resolution of inflammation. Similarly, the nuclear receptors PPARg and PPARd regulate various aspects of M2 macrophage activation and oxidative metabolism. Krüppel-like factors such as KLF4 and KLF2 promote M2 macrophage functions through STAT6 and by suppressing the NF-κB/HIF-1α–dependent pathway. In the same context, c-Myc gets activated by IL-4 and regulates M2-associated genes such as Scarb1, Alox15, Mrc1, STAT6 and PPARg. Epigenetically IL-4 also induces the Jmjd3-IRF4 axis to inhibit M1 polarization through IRF5 and thus promote M2 polarizing conditions. Akt kinases and noncoding RNAs such as mir155 and mir146 have also been shown to regulate macrophage polarization.

The general dogma is that under conditions such as allergy, cancer and parasite infections the functional macrophages phenotypes *in vivo* are similar to those of canonical M1/M2 polarized states, as defined *in vitro*. Importantly, a key concept is that macrophage polarization and thus its activation status has been linked to pathology. M1 polarized macrophages have been implicated in initiating and sustaining inflammation, while the M2 polarized phenotype has been associated with the resolution of inflammation, and if dysregulated, with chronic inflammation. However, macrophage plasticity has been mainly addressed in *in vitro* systems that do not consider the complexity that the macro- and microenvironment provide within the tissue. Thus, considering the observations from recent *in vivo* studies, the general dogma is currently under revision.

- **Resolution of Inflammation**

Arising from these advances, it has been proposed that most of the chronic inflammatory diseases may be caused not only by ongoing pro-inflammatory processes, but also by dysregulated resolution, and thus therapeutic intervention might be very effective if focused in guiding inflammation towards pro-resolution. In general, resolution of inflammation involves the clearance of infiltrated inflammatory cells and restoration to homeostasis. This process is not considered passive but rather tightly regulated events need to occur. Initially, the inflammatory cues (CC-cytokines, CXC chemokines etc.) that triggered inflammation are eliminated, thus preventing further inflammatory cell infiltration and edema formation. At the same time synthesis of pro-inflammatory mediators is inhibited. Next, immune cells of the initial response such as neutrophils and eosinophils are cleared from the inflamed tissue by local apoptosis and subsequent uptake by recruited monocyte-derived macrophages in a process called efferocytosis. Once this process is complete, macrophages either undergo local apoptosis or exit the tissue by lymphatic drainage.

However, it has been recently proposed that there is a third phase called post-resolution. During this phase, there is a second influx of myeloid cells (including $CD11B^{+}CD49d^{+}CD115^{+}MHCII^{+}$ myeloid-derived suppressor cells, $F4/80^{low}MHCII^{+}CD11c^{+}$ DCs, and $F4/80^{mid}CD11B^{hi}CD11c^{-}$) into the tissue and remain for weeks to regulate either the adaptive response or the magnitude and duration of a subsequent acute innate response. This suggests that resolution is connecting innate and adaptive immunity, and chronic inflammation could be explained as incomplete resolution of the initial acute inflammatory response which in turn fails to initiate an appropriate adaptive immune response.

As already mentioned above, to initiate the resolution process, certain events need to take place, (1) removal of stimuli, (2) dampening of pro-inflammatory signaling, and (3) clearance of pro-inflammatory mediators. The importance of antigen clearance is demonstrated in immunodeficiency disorders, such as chronic granulomatous disease, and autoimmune diseases, such as rheumatoid arthritis and systemic lupus erythematosus, that are driven by persistent endogenous antigens. Following stimulus removal, receptors and their signaling pathways that triggered the release of pro-inflammatory mediators must be turned off in order to prevent excessive damage from an ongoing pro-inflammatory state. An example of a failure in dampening of pro-inflammatory signaling is sepsis. During this condition, large amounts of cytokines are still present despite negative bacterial blood cultures. A number of inflammation regulatory mechanisms have been described, such as through miRNAs (i.e. miR-146, miR-21), ARE-binding proteins (tristetraprolin B-related factor 1, KH-type splicing regulatory protein and ARE RNA-binding protein 1), leucine- rich repeat-containing protein 33 (LRRC33) or lipid mediators such as prostaglandin E2 (PGE2), that inhibit IFNg and TLR signaling and subsequently NFkB activation. Following removal of the injurious agent and turning off signaling pathways, the levels of cytokines, chemokines, eicosanoids, cell adhesion molecules and other inflammatory mediators need to be catabolized and return back to their pre-inflamed state. These data indicate that there are various routes to resolution; thus, it is unlikely that one single intervention will be a panacea for all inflammatory diseases.

The development and application of pro-resolution therapeutic strategies to treat chronic inflammation may transform the management of some of the oldest and most frequent human illnesses. By approaching such diverse disease states from the opposite perspective; asking not how to suppress the initiation of inflammation, but how to enhance clearance of the triggering stimuli, we will hopefully gain new insights into both the origin of chronic inflammation and pharmaceutical therapeutics of tackle them.

2. Tissue injury and regeneration

- **Overview**

Tissue repair and regeneration are vital for survival. Repair, also referred to as healing, is the restoration of tissue structure and function following an injury. Infection, toxic or mechanical injury can severely impact tissue integrity. Repair of injured tissues occurs by two types of processes: proliferation of uninjured cells and maturation of tissue stem cells. However, some tissues are able to replace the damaged components and essentially return to a normal state in a process called regeneration. Regeneration occurs by proliferation of cells that survive the injury and retain the capacity to proliferate. For example, in the rapidly dividing epithelia of the skin and intestines, liver and skeletal muscle. However, mammals have a limited capacity to regenerate damaged tissues and organs, and only some parts of most tissues are able to fully regenerate. Notably skeletal muscle, skin and liver have been used as reference tissues for the study of tissue regeneration.

Three types of tissues exist: labile, stable and permanent. (1) Cells from labile tissues are continuously being lost and replaced by maturation from tissue stem cells and by proliferation of mature cells. These cells include the hematopoietic cells in the bone marrow, the majority of surface epithelia (skin, oral cavity, vagina, and cervix), the cuboidal epithelia of the ducts draining exocrine organs (salivary glands,

pancreas, biliary tract), the columnar epithelium of the gastrointestinal tract, uterus, and fallopian tubes; and the transitional epithelium of the urinary tract. These tissues can readily regenerate after injury as long as the pool of stem cells is preserved and provided the underlying basement membrane is intact. (2) Cells from stable tissues are quiescent (in the G0 stage of the cell cycle) and have only minimal proliferative activity in their normal state. These cells can proliferate in response to loss of tissue mass or due to injury. Stable cells constitute the parenchyma of most solid tissues, such as liver, kidney, and pancreas but also endothelial cells, fibroblasts, and smooth muscle cells. Stable tissues have a limited capacity to regenerate after injury except from liver. Mechanisms underlying this response are not understood, but likely involve local production of growth factors and interactions of cells with the ECM. (3) Cells from permanent tissues are considered to be terminally differentiated and non-proliferative in postnatal life. The majority of neurons and cardiac muscle cells belong to this category. Thus, injury to the brain or heart is irreversible and results in a scar, because neurons and cardiac myocytes cannot regenerate. Limited stem cell replication and differentiation occur in some areas of the adult brain, and there is some evidence that heart muscle cells may proliferate after myocardial necrosis. Nevertheless, whatever proliferative capacity may exist in these tissues, it is insufficient to produce tissue regeneration after injury. Thus, in permanent tissues, repair is typically dominated by scar formation. Skeletal muscle is usually classified as a permanent tissue, but satellite cells attached to the endomysial sheath provide regenerative capacity to muscle.

The repair process consists of inflammation and proliferation of satellite and other cells. Wounding causes the rapid activation of coagulation pathways, which results in the formation of a that clot contains fibrin, fibronectin, and complement proteins. The clot serves to act as a scaffold for migrating cells, which are attracted by growth factors, cytokines, and chemokines released into the area. Within 24 hours, neutrophils are seen, migrating toward the fibrin clot. They release proteolytic enzymes that begin to clear the debris and within 48 hours, satellite cells start to proliferate. By day 3, neutrophils have been largely replaced by macrophages which are key cellular constituents of tissue repair, clearing extracellular debris, fibrin, and promoting angiogenesis and ECM deposition. By day 5, neovascularization reaches its peak but these new vessels are leaky, allowing the passage of plasma proteins and fluid into the extravascular space. In addition, migration of fibroblasts to the site of injury is driven by chemokines, TNFa, PDGF, TGF-β, and FGF and their subsequent proliferation is triggered by multiple growth factors, including PDGF, EGF, TGF-β, and FGF, and the cytokines IL-1b and TNFa. During the second week, the leukocyte infiltrate, edema, and increased vascularity are substantially diminished.

- **Animal models and morphological characteristics of acute muscle injury and repair**

Muscle regeneration is a highly efficient process that if compromised can lead to several pathologies such as myopathies. Therefore, understanding the processes of tissue homeostasis, maintenance and regeneration is crucial to devise novel therapeutics. In order to study such processes, the use of reproducible and tightly controlled experimental models of muscle injury and repair are needed. The most commonly used models of acute muscle injury and repair are mechanical injury (freeze injury, irradiation, crush, denervation and transplantation), myotoxins (notexin and cardiotoxin) and chemical agent ($BaCl_2$). Although the regeneration process restores the muscle tissue in all these models, the pathophysiological processes involved, vary depending on the model (differences regeneration kinetics, re-vascularization, and satellite cell behavior). To begin with, CTX, NTX and $BaCl_2$ induce severe necrosis of the tissue, followed immediately by neutrophil and macrophage infiltration that leads to synchronous regeneration. Next myoblasts appear and start fusing to form larger structures (myotubes). The tissue regenerates ad integrum within a relatively short period (8 days in young adult mice). During the freeze-injury model and other mechanical injury models, asynchronous regeneration takes place instead. In these models, there are distinct regeneration foci in which all the different regeneration steps are present at the same time. In addition, although the inflammation kinetics appear to be identical for all models; differences in cytokine

production exist. For example, after CTX injury, inflammatory cytokine expression returned to basal levels when the muscle was morphologically regenerated, while in the other models, the expression of cytokines was never back to basal levels. This suggests that there is still ongoing inflammation in these models despite the normal appearance of the regenerated muscles. Skeletal muscles are comprised largely of differentiated myofibres that have irreversibly left the cell cycle. Following injury, satellite cells which are mostly quiescent, re-enter the cell cycle and generate myoblasts that will participate in muscle restoration.

Skeletal muscle regeneration has emerged as an important paradigm to study the role of satellite and stromal cells following tissue injury. Satellite cells are vital to muscle regeneration but their function involves interactions with the macro- and micro-environment including interactions with macrophages and fibroblasts. The satellite cell microenvironment changes based on the injury model. For example, after NTX or freeze injury, severe vascular lesions and granulomas (multinucleated giant cells) appear which explains the differences in regeneration kinetics. In all of the injury models, loss of satellite cells can be seen, but it also varies based on the severity of the injury. Different volumes of toxins can have an impact on the area of the injured muscle, with higher volumes leading to higher satellite cell death and a delay in their division. In general, in the NTX, CTX and $BaCl_2$ models almost 60% of the satellite cells survive while with freeze injury about 95% of satellite cells are lost. However, 3-4 weeks month post-injury, a higher number of satellite cells can be seen in all models while they continue to cycle at different rates based on the model. Lastly re-vascularization is an important process during skeletal muscle regeneration since it can impact both the distribution of recruited inflammatory cells and regeneration-related factors (growth factors, cytokines, chemokines) but also the communication between endothelial and satellite cells. The mechanical injuries result in a more severe destruction of the vasculature compared to the other models prolonging the regeneration process. Since 85% of satellite cells are located close to blood vessels, it can have an impact on satellite cell oxygen availability, which in turn could affect their proliferation and differentiation.

Muscle regeneration following any acute injury is a very efficient and reliable process to provide fast recovery. On the other hand, recovering from a chronic related damage is not a very efficient process due to the lack of a system adapted to these types of injuries. As a result, immune responses related to muscle regeneration get dysregulated, cause muscle fibrosis and lead to impaired regeneration. Such muscle regeneration defects can be seen for example in Duchenne muscular dystrophy (DMD). In this DMD disorder the cell membrane is weak, causing chronic damage, repeated cycles of regeneration, leading to chronic inflammation that exacerbates the pathology. Importantly, it's been shown that chronic injury alters the macrophage phenotypes responsible for proper muscle regeneration following an acute injury, which in turn disrupt the normal regulation of connective tissue deposition. Furthermore, the repair macrophages associated with dystrophy seem to become highly fibrogenic (by enhancing arginase 1 activity) and exacerbate the fibrosis-related pathology. However, despite the critical role of macrophages in muscular dystrophy, the mechanisms that control the phenotypic transition of macrophages in chronically injured muscle remain elusive.

3. Myeloid cell regulation of skeletal muscle regeneration

- **Overview**

Many cell types are taking part in the regeneration process but resident and recruited macrophages, due to their plasticity, play a vital role in all stages of repair and fibrosis. In fact, a recent cell-depletion study revealed that macrophages are critically required for full limb regeneration in adult salamanders, but surprisingly, wound closure following limb amputation is much less dependent on macrophages. A similar macrophage-depletion strategy in mice determined that macrophages provide critical signals that drive angiogenesis and tissue regeneration after myocardial infarction in neonatal hearts, which are capable of complete regeneration. In the context of muscle regeneration macrophage depletion completely abolishes

the ability of skeletal muscle to regenerate. Importantly, macrophages are also critical to the clearance of senescent cells and owing to their role in controlling the initiation, maintenance and resolution of wound-healing responses in different organ systems, by creating a regeneration-permissive environment, they are considered major therapeutic targets.

Different macrophage populations reside in many tissues and usually derived from the yolk sac during the early stages of embryogenesis. However, some derive from later stages of embryogenesis such as from fetal liver and hematopoietic stem cells. The tissue resident macrophages are important during development but also for normal tissue homeostasis by producing growth and trophic factors to support neighboring tissues. Following acute injury, a large number of inflammatory Ly6C^{high} monocytes are recruited from the bone marrow and circulation, via gradients of chemokines and adhesion molecules. These macrophage precursors far exceed the numbers of tissue resident macrophages, but both proliferate and undergo distinct phenotypic changes in response to the local tissue microenvironment milieu. Early studies on macrophage contribution to tissue repair was focused on their role as scavenger cells that uptake cellular debris, pathogens, neutrophils and other apoptotic cells following tissue injury. However, a number of recent studies have demonstrated a more complex, specialized and critically timed states of monocytes and macrophages during repair, regeneration and fibrosis. Data from these studies support one of macrophage's hallmark characteristic which is plasticity. It appears that different macrophage phenotypes could be tissue-, stimulus- and phase-specific. A tissue such as skeletal muscle can cause distinct populations of immune cells to appear depending on disease etiology. For example, in a skeletal muscle regeneration paradigm, inflammatory monocytes infiltrating the injured muscle switch to a repair macrophage phenotype to support myogenesis in an AMPKa1 (5'-AMP-activated protein kinase catalytic subunit α1) dependent manner. On the other hand, dystrophic muscle and increased pathological fibrosis has also been associated with distinct macrophage populations.

During the initial cellular response following the injury, chemokines, matrix metalloproteinases and various inflammatory molecules are produced by the infiltrating macrophages. This is supported by the fact that if you deplete macrophages early after the injury, the inflammatory response will greatly be diminished. However, as mentioned above, depletion of macrophages will lead to impaired clearance of necrotic debris and subsequently to less efficient regeneration. Following the initial inflammatory phase, macrophages acquire a pro-resolution and tissue repair phenotype which is characterized by the production of several growth factors, such as members of the TGF- β superfamily including transforming growth factor β1 (TGF-β1) and other growth factors such as platelet-derived growth factor (PDGF), insulin-like growth factor 1 (IGF-1), and vascular endothelial growth factor α (VEGF-α). These secreted factors seem to promote satellite and fibroblast cell proliferation, differentiation and neovasculature development. This macrophage subtype can also facilitate the synthesis of extracellular-matrix components, respond and secrete interleukin-10 (IL-10) and other inflammation inhibitory mediators, such as programmed cell death ligands 1 and 2. In addition, the current notion is that monocytes and macrophages respond differently to similar inflammatory signals and according to the inflammation phase, hence showing that diverse phenotypes are neither M1 (pro-inflammatory) nor M2 (anti-inflammatory). In conclusion, different macrophage phenotypes seem to play crucial and unique roles at each stage of the regeneration process and identifying the regulators of the phenotypic shift is critical to devise targeted muscle-inflammation related therapeutics.

- **Inflammatory monocyte to repair macrophage transition**

Mechanisms of infection-induced inflammation are understood far better than those of other inflammatory processes such as injury related. Although infection-induced inflammation is vital, it might be a special case since it is clear now that injury-related physiological inflammation does not seem to fit the classic pattern of inflammation. The reason behind this is that cell states are distinct while tissue states are

graded. For example, tissues might contain cells that are dead, malfunctioned or healthy, all at the same time. Thus, at normal conditions, the tissues remain at homeostatic state, usually by the help of tissue-resident macrophages. However, when tissues undergo stress due to an acute injury for example, they can malfunction if the insult is excessive. In this case additional recruited macrophages are needed to help the tissue overcome these issues. A particularly interesting and important aspect of regeneration is the invasion and conversion of circulating inflammatory monocytes into inflammatory macrophages (MFs) and then conversion into repair-type ones. Acute muscle injury causes the recruitment of monocytes/macrophages into the damaged area of the muscle and this invasion peaks within the first 2 days after the injury. In the absence of macrophages, muscle regeneration is severely impaired, highlighting the importance of these cells in the regeneration process. The roles of these immune cells in response to injury are to (1) confine the damage, (2) clear the necrotic debris through phagocytosis, and (3) repair the damage. There are multiple lines of evidence to suggest that macrophages instruct satellite cells to proliferate and differentiate, by secreting cytokines, growth factors and other molecules that regulate the satellite cell pool, including our recent findings that macrophage-secreted growth and differentiation factor 3 contributes to myoblast fusion. This highly dynamic process is characterized by an in-situ transition of infiltrating monocytes from an inflammatory ($Ly6C^{high}F4/80^{low}$) to a repair ($Ly6C^{low}F4/80^{high}$) macrophage phenotype, which appears to be indispensable for proper muscle regeneration.

Distinct inflammatory and repair macrophage phenotypes have also been reported in spinal-cord injury models. In these models, inflammatory monocytes ($Ly6C^{high}CX3CR1^{low}$) enter the injury sites in a CCL2-depentent manner, whereas repair macrophages ($Ly6C^{low}CX3CR1^{high}$) movement is controlled by adhesion proteins (VCAM-1, VLA-4) and endothelial-cell-surface enzymes that are implicated in leukocyte extravasation. Thus, it appears that the guided trafficking of infiltrating monocytes and macrophages towards the injury site provides crucial signals to shape their unique functions. Similar studies performed in other tissues, revealed regulators (such as regenerating islet-derived 3β and C19orf10) of macrophage movement to injured cardiac muscles. These factors are involved in removal of neutrophils by repair macrophages, thus protecting these tissues from extensive damage and cardiac rupture following myocardial infarction. Recent studies also highlight that a single population of monocytes can acquire both pro-inflammatory and pro-repair phenotypes, suggesting that an *in situ* phenotypic transition, rather than recruitment of a different pro-reparative Ly6C subset, takes place. For example, removing the $Ly6C^{-}$ monocytes populations from the circulation does not affect the presence of repair macrophages or the outcome of muscle regeneration. In addition, activin-a (supports oligodendrocyte differentiation) has been identified as a repair macrophage-derived mediator participating in the phenotypic shift of microglia from a pro-inflammatory to a repair phenotype. Similar findings in liver injury models suggest that cytokines such as IL-4, IL-10, and the uptake of dead cells through phagocytosis regulate the conversion of inflammatory monocytes into repair macrophages. In addition, in these models Wnt signaling appears to be an important pathway in macrophages, which regulates regeneration by driving the differentiation of progenitor cells to hepatocytes. In a lung injury model (acute pathogen-induced injury), it has been shown that IL-17, IL-4, IL-10 and IGF-1 drive the conversion to an anti-inflammatory macrophage phenotype to promote lung healing. In the context of muscle regeneration, IL-10 also seems to play a key role in the phenotypic switching of muscle macrophages through CCAAT/enhancer binding protein beta (C/EBPb). Even though macrophage invasion is not altered in the C/EBPb null mice M2-specific genes are altered leading to impaired regeneration. However, it is important to note that recent studies have questioned the importance of M2-like macrophages and IL-4 in muscle regeneration. Nonetheless, repair macrophages directly target satellite cells by secreting cytokines and growth factors to promote their differentiation into mature myotubes. On the other hand, inflammatory macrophages inhibit myogenic precursor fusion demonstrating again that different macrophage states can have various effects on satellite cell fate and subsequently muscle regeneration. Together, these studies, demonstrate how the timely transition of inflammatory monocytes to reparative macrophages is critical for tissue regeneration.

The macrophage phenotype shift involves many other molecular and cellular processes. Phagocytosis of neutrophils for instance, controls the conversion and in case phagocytosis is impaired macrophages cannot acquire the repair phenotype. Similar to acute injury models in other tissues, cytokines orchestrate the conversion in muscle macrophages. IFN-g promotes the inflammatory phenotype while inhibiting the repair one. In support of this observation an increase in repair macrophage activation and a decrease in muscle damage have been observed in IFN-g null mice. At the cellular level mitogen-activated protein kinase phosphatase-1 (MKP-1), controls the phenotypic shift of muscle macrophages through regulating mitogen-activated protein kinase (MAPK) p38 and Akt kinases. In MKP-1 null mice the transition occurs faster resulting in dysregulated regeneration. As mentioned in the previous section the involvement of AMPK-a1 in the phenotypic transition has also been demonstrated. AMPK-a1 null mice express inflammatory cytokines at higher levels while the AMPK-a1 overexpression model reveals a significant decrease in their expression. These studies show that the conversion of muscle macrophages from inflammatory to repair ones is finely regulated with multiple targets and pathways being involved. How all these elements are regulated and coordinated is still unclear.

- **Effector molecules and secreted growth factors in the regulation of muscle regeneration.**

Interest in deciphering the pathways and the diverse macrophage subsets that sustain tissue-damaging inflammatory responses has increased lately, given that delineating the mechanisms would lead to developing novel therapeutics for various types of inflammatory diseases. Following acute sterile muscle injury, macrophages secrete cytokine to activate and enhance satellite cell proliferation and differentiation. At the same time, the tissue itself produces mediators and factors (myokines) that act on an autocrine or endocrine manner and influence the regeneration process. Most of the studies identifying these myokines where performed in the context of physical activity but recently some of these factors have been discovered in the context of muscle regeneration and seem to heavily impact this process. For example, human myoblasts secrete IL-6 in response to IL-1, TNF-a or LPS treatment *in vitro* and in IL-6 null mice migration and proliferation of satellite cells is greatly diminished. In addition, since infiltrating macrophages also secrete IL-6 they further stimulate proliferation of satellite cells. In C2C12 myocyte cell cultures, IL-6 has been shown to be involved in the myocyte differentiation program as well. Inhibiting IL-6 decreased differentiation while overexpression increased myotube formation. Overall, these results highlight IL-6 as a myokine secreted by multiple sources including macrophages and impact satellite cell migration to the injury site, proliferation and differentiation into myotubes. In a recent study from our lab and as part of this dissertation we identified growth differentiation factor 3 (GDF3), a secreted factor and a member of the transforming growth factor-β family, that already includes known regulators of muscle regeneration, as a potent inducer of myotube formation.

In a similar fashion, leukemia inhibitory factor (LIF) is another myokine secreted in the muscle during exercise and regeneration. Interestingly, *in vivo* infusion of LIF enhances regeneration by directly acting on proliferation and differentiation of satellite cells. *In vitro*, LIF activates C2C12 maturation through the JAK2/STAT2 pathway, while targeting of the LIF receptor with siRNA markedly decreases human myoblast proliferation. In human skeletal muscle interleukin 7 (IL-7) seems to have similar function on satellite cell differentiation and similarly targeting the IL-7 receptor decreases their maturation to myotubes *in vitro*. In addition, recombinant IL-7 treatment activates satellite cell migration but inhibits their maturation. Thus, in the context of muscle injury and regeneration, IL-7 is involved guiding the satellite cells to the injury site and keeps them from differentiating until the proper signals overcome this inhibition. Interleukin 4 is another cytokine proposed to affect satellite cell differentiation and myotube formation through fusion. CXC ligand 1 (CXCL-1) and interleukin 8 (IL-8) are another group of myokines that impact regeneration by attracting neutrophils to the injury site, although their exact mode of action in skeletal muscle regeneration is still not clear. During aging, production of these cytokines/myokines is either diminished or altered,

leading to declining muscle function. Thus, it will be interesting for future studies to address the potential effect these molecules could have in restoring the age-related muscle repair inefficiency.

- **Muscle resident-immune cell cross-talk during regeneration**

Skeletal muscle regeneration is a collection of highly synchronized processes involving several cellular, molecular and signaling responses. Coordinating inflammation and regeneration is particularly important for efficient repair (ad integrum) following acute injury. A chief reason for the lack of better understanding of muscle repair is that skeletal muscle tissue comprises many different cell types, such as satellite cells, fibroblasts, fibro/adipogenic progenitors (FAPs), endothelial and periendothelial cells, mast cells, macrophages (resident and patrolling circulatory monocytes) and T cells, which can all affect the myofiber environment. Any disturbance of these cells' interactions could disrupt tissue homeostasis and consequently repair. Satellite cells are known to be key regulators of the skeletal muscle compartment. These cells are undifferentiated myogenic cells that are found between the sarcolemma and basal lamina, and can fuse to establish new myofibres. These quiescent cells mature into post mitotic myofibres through specific extracellular signals. Initially these paracrine or autocrine signals result in myoblast expansion followed by pro-differentiation signals that are required to induce their maturation. The exact nature of such signals is still not clearly defined. Many candidate molecules have been proposed to mediate myoblast differentiation such as IL-6, insulin growth factors, and more recently growth differentiation factors, supporting the notion that a range of factors are involved in this differentiation process. Furthermore, only recently we started to understand which cell types (FAPs and macrophages) are responsible for producing these factors. In the context of muscle regeneration following acute sterile injury, the satellite cells interact primarily with inflammatory and stromal cells, i.e. fibroblasts, pericytes and FAPs. Importantly, it has been suggested that the cross-talk between these tissue compartments is crucial both in regulating myoblast expansion and differentiation and regarding the outcome of skeletal muscle regeneration. However, it is not known how cross-talk between satellite cells and other tissue compartments contributes to synchronized repair. At the same time, the field lacks adequate methodology to study and thoroughly delineate the exact nature of such interactions.

Nonetheless, it is known that repair macrophages activate angiogenesis and induce satellite cell differentiation. A model of repair macrophage depletion (CCR2 −/− mice), shows the importance of this subtype in regeneration, while *in vitro* studies demonstrate that these cells enhance the expression of myogenin (muscle differentiation marker), and myocyte fusion. In human muscle biopsies after exercise, it is being shown that the repair macrophages co-localize with differentiating satellite cells. Insulin-like growth factor-1 (IGF-1) is an important hormone secreted by the repair macrophages acting on satellite cells and seems to restore proper regeneration dynamics when administered exogenously. With aging satellite cell proliferation and differentiation capacity declines, as well as macrophage cytokine production. Knowing the key role of macrophages in regulating satellite cell function, one can assume that changes due to aging affects muscle regeneration. However, it's still unclear how aging affects satellite cell function and macrophage phenotypic transition, and their subsequent immune/muscle cell crosst-talk. Such work will help us to develop approaches to preserve the muscle regenerative capacity in aged individuals.

Aims

Our work focused on identifying multi-level interactions between inflammatory and repair macrophages and skeletal muscle cells during regeneration and repair. Our long-term goal has been to identify and characterize sensory, regulatory and effector functions ascribable to macrophages during muscle regeneration and tissue repair. We have used unbiased genomic approaches (RNA-seq and ATAC-seq) combined with advanced cell separation from injured and regenerating tissues to identify molecules likely to be required as sensors of the environment, regulators of transcription during the macrophage phenotypic switch, and effector molecules contributing to myoblast proliferation, differentiation and myofiber formation.

Specific aims:

1. Assessing the role of newly identified sensors and regulators in a model of skeletal muscle regeneration (CTX):
   a) Lipid activated transcription factor PPARg:
      - *In vivo* molecular analysis of macrophage PPARg function in inflammation and tissue injury (fiber size distribution, necrotic clearance).
      - Identification of PPARg target genes using gene expression profiling and characterization of the contribution of these genes to muscle regeneration.

   b) Heme sensor and transcriptional repressor BACH1:
      - *In vivo* molecular analysis of macrophage BACH1 function in macrophage dynamic phenotype switch, cell invasion, gene expression, inflammation and tissue injury (fiber size distribution, necrotic clearance).
      - Identification of BACH1 target genes using gene expression profiling and characterization of the contribution of these genes to muscle regeneration.

2. Assessing the role of newly identified effector molecules in an acute sterile muscle injury model (CTX):
   a) Growth differentiation factor GDF3:
      - *In vivo* molecular analysis of macrophage secreted GDF3 in muscle injury (fiber size distribution).
      - GDF3 role in primary myoblasts and aged muscles (*in vivo* administration of recombinant protein).

   b) Growth differentiation factor GDF15:
      - *In vivo* molecular analysis of macrophage GDF15 function in macrophage dynamic phenotype switch, cell invasion and tissue injury (fiber size distribution).

   c) Heme catabolizing enzyme HMOX1:
      - *In vivo* molecular analysis of macrophage HMOX1 in macrophage dynamic phenotype switch, cell invasion, and tissue injury (fiber size distribution, necrotic clearance).

3. Explore the potential existence of a reciprocal cross-talk between the skeletal muscle tissue and myeloid cells recruited following acute injury:
   a) Use two *in vivo* models as a mean to alter cellular composition prior to muscle injury:
      - Irradiation.
      - Genetic ablation of a specific muscle cell population (PAX7$^+$ cells).

   b) Use bone marrow transplantation to replenish myeloid cells.
   c) Use PET-MRI *in vivo* imaging to monitor the myeloid cell phenotypic switch *in situ*.

Materials and Methods

Ethical approval:

All animal experiments were carried out in accordance with guidelines prescribed by the Institutional Animal Care and Use Committee at Sanford Burnham Prebys Medical Discovery Institute and University of Debrecen, School of Medicine following Hungarian (license no.: 21/2011/DEMÁB) and European regulations.

Mice:

Wild type BoyJ (B6.SJL-Ptprca Pepcb /BoyJ, stock number 002014) and C57BL/6J male control mice were obtained from the Jackson Laboratories and bred under specific-pathogen free (SPF) conditions. $Pparg^{fl/fl}$LysMCre+ (refered to as PPARg MacKO), Gdf15 KO (provided by Dr. Se-Jin Lee at John Hopkins University), Bach1 KO (obtained by Dr. Kazuhiko Igarashi at Tohoku University), $Hmox1^{fl/fl}$ LysMCre (obtained by Dr. George Kollias at BSRC-Athens), Gdf3 KO (provided by Dr. Chester Brown at Baylor College of Medicine), and littermate C57BL/6 albino controls were used in the experiments. Mice of 23-28 month-old were obtained by GlaxoSmithKline. Experiments were conducted on adult (2-6 month-old) male mice unless otherwise noted. For *in vivo* heme treatments mice were treated with either 30 mg/kg hemin (Sigma) or saline daily. All irradiation experiments were performed under anesthesia in cohorts of 12 animals per experiment. Briefly, mice were anaesthetized with a single intraperitoneal dose of ketamine/xylazine (ketamine 80-100 mg/kg, xylazine 10-12.5 mg/kg). Irradiated and bone marrow transplanted mice were maintained in an SPF status (autoclaved top filter cages) for the entire course of experimentation and antibiotics (amoxicillin antibiotic, clavulanic acid [500mg/ 125 mg/ liter of drinking water]) were administered in the drinking water for 4 weeks after transplantation to minimize bacterial contamination within the water source and potentially decrease the burden of gastrointestinal bacteria. Irradiated mice were also fed autoclaved rodent chow ad libitum. Animals that undergo irradiation for BMT typically lose a considerable amount of weight, only to gain it back relatively quickly after successful transplantation. At our institutions, weight loss of 20%, or greater was used as a rationale for euthanasia before the intended experimental endpoint according to the IACUC guidelines. The Pax7CreER/+, Rosa26DTA/+ strain, designated Pax7-DTA, was generated by crossing Pax7CreER/CreER and Rosa26DTA/DTA strains. Pax7-DTA pups were genotyped by PCR are as previously described (Nishijo, Hosoyama et al. 2009), (Wu 2006). The tamoxifen-treated mice were kindly provided by Dr. John McCarthy and Dr. Charlotte Peterson. When necessary and for tissue collection mice were euthanized by either isoflurane overdose (adjusted flow rate or concentration to 5% or greater) or CO2 exposure (adjusted flow rate 3 L/min) in accordance to the University of Debrecen and Sanford Burnham Prebys Medical Discovery Institute's IACUC guidelines.

Bone Marrow Transplantation (BMT):

Recipient congenic BoyJ mice (7 weeks old) are irradiated with 9.5 or 11 Gy using a Theratron 780C cobalt unit for the ablation of the recipient bone marrow. The animals to be irradiated were immobilized using a circular cage (mouse pie cage) that can hold up to 11 mice (alert mice). Following the irradiation, isolated bone marrow cells (in sterile RPMI-1640 medium) flushed out from the femur, tibia and humerus were transplanted into the recipient mice by retro-orbital injection ($20x10^6$ BM cells per mice). This experimental BMT CD45 congenic model allows us to detect donor, competitor and host contribution in hematopoiesis and repopulation efficiency of donor cells (congenic mice with CD45.1 versus CD45.2). The CD45.1 and CD45.2 contribution is detected by flow cytometry at 8-12 weeks following the BMT. In short, a cut at the tail tip of the mice provided a drop of blood that was placed into 0.5 ml PBS + 1% FBS + 10 U/ml Heparin buffer (samples kept on ice). The cells were directly stained by 2 µl (1/50 dilution) mouse anti-mouse

CD45.2-FITC (clone 104) and 2 µl (1/50 dilution) rat anti-mouse GR1-PE (clone RB6-8C5) antibodies (BD Pharmingen) and incubated on ice for 30 min. After 2 washes with ice-cold PBS/FBS/Heparin buffer, cells were resuspended in 0.5-1 ml FACS Lysing solution (BD Cat #349202). Cells where then incubated for 5 min at RT and centrifuged (400g, 5 min, 4°C). The double stained samples were run on BD FACS Calibur and determined the ratio of donor cells. The repopulation is always gated on the granulocyte fraction based on FSC, SSC and Gr1 staining.

Bone Marrow Transplantation (BMT) including local radioprotection:
A modification of the above protocol was used by introducing hindlimb lead shielding. The animals to be irradiated were anesthetized via intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg), and placed into a plastic box, with double wall. Water was used between the walls, as scattering medium, in order to get homogeneous dose distribution. Two 5 cm high led blocks were used for protection one of the legs of the mouse. The blocks were on the PMMA shadow tray of the cobalt unit, more than 20 cm distance to the surface of the plastic box, so the air absorbs the Compton electrons from the blocks. In order to account for the buildup region of cobalt energy, 5 mm thickness top box plate was used. The source surface distance (SSD) and source axis distance (SAD) was 80 cm and the beam size covered 21 x 21 cm. The activity of the Cobalt-60 isotope was about 50 TBq (1350 Ci). Animals were generally irradiated for short periods of time (20-30 min as a function of the given date) depending on the radioisotope decay charts, amount of irradiation needed, and source of ionizing energy. Following the irradiation, isolated bone marrow cells from donor C57BL/6J mice were transplanted into the recipient mice by retro-orbital injection.

Lower body irradiation:
For this method 5 cm thick lead blocks were used during irradiation, to protect one of the back legs of lightly sedated mice. We focused the radiation field (14.4 x 4.8 cm) only in the lower part of their bodies just below the hip thus minimizing radiation exposure to the rest of the body. The source surface distance (SSD) and source axis distance (SAD) was kept the same and mice were irradiated with a dose of 11 Gy. The animals left to recover for 8 weeks after the radiation exposure.

Acute sterile muscle injury:
Mice were anaesthetized with isoflurane (adjusted flow rate or concentration to 1.5 %) and 50 µl of cardiotoxin ($12X10^{-6}$ M in PBS) (from Latoxan) was injected in the tibialis anterior (TA) muscle. Muscles were isolated for flow cytometry analysis at day 1 to day 8 post-injury or for muscle histology at day 8 to day 20 post-injury.

Histological analysis of muscle regeneration:
Muscles were removed and snap frozen in nitrogen-chilled isopentane (–160°C). 8 µm thick cryosections were cut and stained with hematoxylin-eosin (H&E). For each histological analysis, at least 5 slides (per condition) were selected where the total regenerative region within the CTX injured TA muscle was at least 70%. For each TA, myofibers in the entire injured area were counted and measured. H&E muscle sections were scanned with Mirax digital slide scanner and the Cross Sectional Area (CSA) was measured with HALO software (Indica Labs). CSAs for these samples are reported in µm2. Quantitative analysis of necrotic/phagocytic vs. centrally nucleated myofibers was performed using the Panoramic Viewer software and was expressed as a percentage of the total number of myofibers. Necrotic/phagocyted myofibers were defined as pink pale patchy fibers that are invaded by basophil single cells (macrophages).

Immunohistochemistry (Frozen sections):
Tissue sections were fixed and permeabilized in ice cold acetone for 5 min and blocked for 30 minutes at 20 °C (room temperature) in PBS containing 2 % bovine serum albumin (BSA). Tissues were stained for 1 h at

room temperature using a primary antibody diluted in 2 % BSA. The primary antibodies used for immunofluorescence were rabbit anti-laminin (L9393 SIGMA) at a dilution of 1/200, chicken anti-PAX7 (DSHB) at a dilution of 1/20, rabbit anti-Desmin (Abcam 32362) at a dilution of 1/200 and rat anti-F4/80 (Abcam 6640) at a dilution of 1/200. In all cases, the primary antibody was detected using secondary antibodies (dilution 1/200) conjugated to FITC (JIR 703-095-155) or Cy3 JIR (711-165-152). The nuclei were counter stained with 0.1-1 µg/ml Hoechst. Fluorescent microscopy was performed using Carl Zeiss Axio Imager Z2 microscope equipped with lasers at 488, 568 and 633 nm. Figures were analyzed and assembled using Fiji (image processing package based on ImageJ) and Illustrator CS5 (Adobe).

In vivo Isolation of macrophages from muscle:

Fascia of the TA was removed. Muscles were dissociated in RPMI containing 0.2% collagenase B (Roche Diagnostics GmbH) at 37°C for 1 hour and filtered through a 100 µm and a 40 µm filter. CD45+ cells were isolated using magnetic sorting (Miltenyi Biotec). For FACS, macrophages were treated with 5% Fcγ receptor blocking antibodies and with 10% normal rat serum: normal mouse serum 1:1 mix, then stained with a combination of PE-conjugated anti-Ly6C antibody at a dilution of 1/200 (HK1.4, eBioscience), APC-conjugated F4/80 antibody at a dilution of 1/50 (BM8, eBioscience) and FITC-conjugated Ly6G antibody at a dilution 1/100 (1A8, Biolegend). Ly6C^{high} F4/80^{low} macrophages, Ly6C^{low} F4/80^{high} macrophages and Ly6G^{high} Ly6C^{med} F4/80^{-} neutrophils were quantified. In each experiment, control samples were processed in parallel to minimize experimental variation. Cells were analyzed on a BD FACSAria III sorter and data analysis was performed using BD FACSDIVA (BD Biosciences) and FlowJo V10 (FlowJo, LLC) software.

RNA isolation:

Total RNA was isolated with TRIZOL reagent according to the manufacturer's recommendation. 20ug glycogen (Ambion) was added as carrier for RNA precipitation.

RT-qPCR:

Transcript quantification was performed by quantitative real-time RT (reverse transcriptase) PCR (polymerase chain reaction) using SYBR Green assays. RT-qPCR results were analyzed with the standard delta Ct method and results were normalized to the expression of Rps26 or Ppia.

Small animal PET-MRI imaging using ^{18}F-FDG:

PET-MRI experiments were conducted during the light cycle to minimize muscle activity. C57BL/6 or BoyJ mice were injected with 8.0 ± 0.2 MBq of ^{18}F-FDG via the lateral tail vein in 200 µl volume. 50 min after ^{18}F-FDG injection animals were anaesthetized by 3% isoflurane with a dedicated small animal anesthesia device and whole body PET scans (10 min static PET scans) were acquired using the preclinical nanoScan PET/MRI system (Mediso Ltd., Hungary). To prevent movement, the animals were fixed to a mouse chamber (MultiCell Imaging Chamber, Mediso Ltd., Hungary) and positioned in the center of field of view (FOV). For the determination of the anatomical localization of the organs and tissues, T1-weighted MRI scans were performed (3D GRE EXT multi-FOV; TR/TE 15/2 ms; FOV 70 mm; NEX 2). PET volumes were reconstructed using a three-dimensional Ordered Subsets Expectation Maximization (3D-OSEM) algorithm (Tera-Tomo, Mediso Ltd., Hungary). PET and MRI video and images were automatically co-registered by the PET/MRI instrument's acquisition software (Nucline). Reconstructed, reoriented and co-registered images were further analyzed with InterView™ FUSION (Mediso Ltd., Hungary) dedicated image analysis software.

PET data analysis:

Radiotracer uptake was expressed in standardized uptake values (SUVs). Ellipsoidal 3-dimensional Volumes of Interest (VOI) were manually drawn around the edge of the TA and GAST muscle activity using InterView™ FUSION multi-modal visualization and evaluation software (Mediso Ltd., Hungary). The

standardized uptake value (SUV) was calculated as follows: SUV = [VOI activity (Bq/mL)]/[injected activity (Bq)/animal weight (g)], assuming a density of 1 g/mL

Ex vivo radiotracer uptake studies:
Cells were isolated from injured muscles as described above. Live cells for both myeloid $CD45^+$ and non-myeloid CD45- fractions were selected using FACS-sorting based on FSC/SSC gating and then washed and resuspended in PBS containing 1 mM glucose. The samples were pre-incubated at 36 °C for 10 min at a cell concentration of 1×10^6 /ml in PBS and 0.37 MBq/ml ^{18}F-FDG was then added to each sample. After the addition of the radioligand, cells were further incubated at 36 °C for 50 min and the uptake was terminated by the addition of ice-cold PBS. The cells were then washed three times with cold PBS and resuspended in 1 ml of cold PBS, and the radioactivity was measured. The tubes were measured in a Canberra Packard gamma-counter for 1 min within the ^{18}F-sensitive energy window. Decay-corrected radiotracer uptake was expressed as counts min^{-1} $(10^6 \text{ cells})^{-1}$ (cpm). The displayed data are the means ± SD of the results of at least three independent experiments, each performed in triplicate.

Expression data processing and analysis:
GSE71155 data sets were loaded into the Genespring GX software, and multiarray average summarization was carried out. Next, the lowest 5% of the entities with detectable signals were filtered out as not expressed. Duplicate entities, not/poorly annotated transcripts and transcripts reporting inconsistent expression values were also discarded. Further analysis was carried out on the filtered data set based on the RAW expression values. Heatmap was generated based on log_{10}-transformed raw values with R software package pheatmap. Hierarchical clustering analysis was then applied by Euclidean distance measure and Ward's clustering algorithm, to find correlated genes.

Myogenic precursor cell (MPC) culture:
Murine MPCs were obtained from TA muscles and cultured using standard conditions in DMEM/ F12 (Gibco Life Technologies) containing 20% FBS and 2% G/Ultroser (Pall Inc). For proliferation studies, MPCs were incubated for 1 day with conditioned medium + 2.5% FBS or with 2.5% FBS medium containing 100-600 ng of GDF3 or GDF15 mouse recombinant protein. Cells were then incubated with anti-Ki67 antibodies at a dilution of 1/200 (15580 Abcam), which were subsequently visualized using Cy3-conjugated secondary antibodies at a dilution of 1/200 (Jackson Immunoresearch Inc). For differentiation studies, MPCs were incubated for 3 days with conditioned medium containing 2% horse serum or with 2% horse serum medium containing 300 ng/ml recombinant GDF3. Cells were then incubated with anti-Desmin antibodies at a dilution of 1/200 (32362 Abcam), and in combination with a Cy3-conjugated secondary antibody at a dilution of 1/200 (Jackson Immunoresearch Inc).

Image capture and analysis for myoblast cultures:
Fusion index (for myogenic cells) was calculated as the number of nuclei within myotubes divided by the total number of nuclei. The nuclei number was estimated based on DAPI staining and using the Image J software. Desmin staining was used for detecting the myotubes.

Microarray analysis of muscle macrophages:
Global expression pattern was analyzed on Affymetrix GeneChip Mouse Gene 1.0 ST arrays. The microarray data are publicly available (Data access: GSE71155).

Western Blotting:
GDF3 and GDF15 protein expression was measured using Western Blot analysis. Samples from CTX injected TA muscles were lysed in RIPA buffer. GDF3 was targeted using rabbit monoclonal Anti-GDF3 primary

antibody (ab109617, Abcam, Cambridge, MA) at 1:1000 dilution in 5% BSA/TBS-T overnight at 4°C. GDF15 was targeted using rabbit polyclonal (ab105738, Abcam) at 1:1000 dilution in 5% BSA/TBS-T overnight at 4°C. Anti-GAPDH mouse monoclonal primary antibody (AM4300, Ambion, Carlsbad, CA) was used as a protein loading control at 1:10000 – 1:20000 dilution in 5% BSA/TBST overnight at 4°C.

RNA sequencing (RNA-Seq) library preparation:

cDNA library for RNA-Seq was generated from 100-400 ng total RNA using TruSeq RNA Sample Preparation Kit (Illumina, San Diego, CA, USA) according to the manufacturer's protocol. Briefly poly-A tailed RNA molecules were pulled down with poly-T oligo attached magnetic beads. Following purification, mRNA was fragmented with divalent cations at 85°C, and then cDNA was generated by random primers and SuperScript II enzyme (Life Technologies). Second strand synthesis was performed followed by end repair, single `A` base addition and ligation of barcode indexed adaptors to the DNA fragments. Adapter specific PCRs were performed to generate sequencing libraries. Libraries were size selected with E-Gel EX 2% agarose gels (Life Technologies) and purified by QIAquick Gel Extraction Kit (Qiagen). Libraries were sequenced on HiSeq 2500 instrument. Three biological replicates were sequenced for each population.

RNA-seq analysis:

RNA-seq samples were analyzed according to the pipeline described in Barta et al. 2011. Briefly, the 50 bp raw single reads were aligned using TopHat (Kim et al. 2013) to the mm10 genome assembly (GRCm38) and only the uniquely mapped reads were kept using '--max- multihits 1' option, otherwise the default parameters were used. SAMtools (Li et al. 2009) was used for indexing the alignment files. Coverage density tracks (wig files) for RNA-seq data were generated by igvtools with 'count' command and then converted into tdf files using 'toTDF' option. Genes with CPM (counts per million) >= 10 (at least in one sample) were considered to be expressed. Statistically significant difference was considered as p value < 0.05 from GLM test using R package edgeR (Robinson, McCarthy et al. 2010). Data analysis was also performed using Strand NGS software, Version 2.8, Build 230243 (Strand Life Sciences). Aligned reads were loaded into Strand NGS before performing DESeq normalization for hierarchical clustering analysis (Euclidean similarity measure and Ward's linkage rule used), and data visualization using Principal Component Analysis (PCA). When comparing data across three or more groups, the obvious option is to use the One-way ANOVA statistical test. The One-Way ANOVA takes a comprehensive approach in analyzing data and attempts to extend the logic of t-tests to handle three or more groups concurrently. It uses the mean of the sum of squared deviates (SSD) as an aggregate measure of variability between and within groups. It assumes independent and random samples drawn from a normally distributed source. Additionally, it also assumes that the groups have approximately equal variances, which can be practically enforced by requiring the ratio of the largest to the smallest group variance to fall below a factor of 1.5. These assumptions are especially important in case of unequal group-sizes. When group-sizes are equal (samples are independent and random) like in the CTX model, the test is amazingly robust, and holds well even when the underlying source distribution is not normal. The significant ANOVA result suggests rejecting the null hypothesis H0 = ''means are the same''. It does not tell which means are significantly different. For a given gene, if any of the group pair is significantly different, then in ANOVA test the null hypothesis will be rejected. Post hoc tests are multiple comparison procedures commonly used on only those genes that are significant in ANOVA F-test. The most common post hoc test is Tukey's Honestly Significant Difference or HSD test. Tukey's test calculates a new critical value that can be used to evaluate whether differences between any two pairs of means are significant. One simply calculates one critical value and then the difference between all possible pairs of means. Each difference is then compared to the Tukey critical value. If the difference is larger than the Tukey value, the comparison is significant. Next multiple testing correction using Bonferroni and Benjamini Hochberg methods (replicate analysis) were used for identifying differentially expressed genes under different experimental conditions. $\log_{10}$ adjusted p-values and $\log_2$ fold changes generated by

these analyses were plotted using R package pheatmap (version 3.2.1) to create heatmaps. Genes with normalized expression lower than 6 across all samples were not expressed and thus removed from the analysis. Pathway analysis was performed with the DAVID (Database for Annotation, Visualization and Integrated Discovery) online tool (https://david.ncifcrf.gov/).

Gene ontology analysis:
Lists of genes were analyzed using Panther tool (http://www.geneontology.org/) and the GO Enrichment Analysis to create a gene ontology (GO). GOs with p values < 0.0001 and fold enrichment > 2 were selected and results were presented according to their $-\log_{10}$(p value).

Differentiation of bone marrow-derived macrophages:
Isolation and differentiation of BMDMs were performed as described earlier (Daniel, Balint et al. 2014). Briefly, isolated bone marrow- derived cells were differentiated for 6 days in the presence of L929 supernatant.

ChIP (Chromatin immunoprecipitation):
ChIP-qPCR was performed essentially as previously described (Daniel, Balint et al. 2014), (Daniel, Nagy et al. 2014). a-Bach1 antibody was a gift from Dr Spilianakis (IMBB-FORTH, Greece).

Real-Time Quantitative PCR for enhancer RNA (qPCR):
RNA was isolated with Trizol reagent (Ambion). RNA was DNase-treated and reverse transcribed with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to manufacturer's protocol. Transcript quantification was performed by qPCR reactions using SYBR green master mix (BioRad). Transcript levels were normalized to Ppia or Actb.

ATAC-seq:
ATAC-seq was carried out as described earlier with minor modification (Buenrostro, Giresi et al. 2013). 20 000 cells were sorted in ice-cold PBS. Nuclei were isolated with ATAC-Lysis Buffer (10mM Tris-HCl pH7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.1% IGEPAL) and were used for tagmentation using Nextera DNA Library Preparation Kit (Illumina) from 2-3 biological replicates. After tagmentation DNA was purified with MinElute PCR Purification Kit (Qiagen). Tagmented DNA was amplified with Kapa Hifi Hot Start Kit (Kapa Biosystems) using 9 PCR cycles. Amplified libraries were purified again with MinElute PCR Purification Kit. Fragment distribution of libraries was assessed with Agilent Bioanalyzer and libraries were sequenced on a HiSeq 2500 platform.

ATAC-seq analysis:
Two replicates of Ly6Chigh and low blood monocytes, muscle-derived Ly6Chigh macrophages 1 day and Ly6Chigh and low macrophages 2 and 4 days upon muscle injury were used for the ATAC-seq experiments (20000 sorted cells per sample). The primary analysis of ATAC-seq-derived raw sequence reads has been carried out using our newest version of ChIP-seq analysis command line pipeline (Barta, 2011) including the following steps: Alignment to the mm10 mouse genome assembly was done by the BWA tool (Li and Durbin 2009), and BAM files were created by SAMTools (Li, Handsaker et al. 2009). Signals (peaks) were predicted by MACS2 (Zhang, Liu et al. 2008), and artifacts were removed according to the blacklist of ENCODE (ENCODE Project Consortium, 2012). All regions derived from at least any two samples were united within 0.5kb and those summits having the highest MACS2 peak score in any sample were assigned to each region. Promoter-distal regions were selected excluding the TSS+/-0.5kb regions according to the mouse GRCm38.p1 (mm10) annotation version. Tag directories used by HOMER in the following steps were generated with a 120-nucleotide fragment length with makeTagDirectory (Heinz, Benner et al. 2010). Genome coverage (bedgraph and tdf) files were generated by makeUCSCfile.pl (HOMER) and igvtools,

respectively, and used for visualization with IGV2 (Thorvaldsdottir, Robinson et al. 2013). Read distribution (RD) heat maps were created by annotatePeaks.pl (HOMER), went through an upper decile normalization and were visualized by Java TreeView (Saldanha 2004). 10 clusters of promoter-distal open chromatin regions were discriminated by K-means clustering based on the RD of all samples used. Dynamically changing clusters with similar profile but different density, were united. Motif enrichments for all 10 clusters were determined by findMotifsGenome.pl (HOMER). Top PU.1 and TRE motif hits as well as the MARE (mafa, mafk and bach1) motifs of HOMER's motif collection were used to map the putative regulatory elements to each region around the chosen summits plotted in 30-nucleotide windows using annotatePeaks.pl (HOMER) and other command line programs including intersectBed (BEDtools) (Quinlan and Hall 2010). TRE motif matches overlapping with MAREs were excluded from the analysis. Motif distribution plots were visualized by Java TreeView.

Statistics:

ANOVA with Bonferroni correction for multiple testing was used to determine statistical significance using GraphPad Prism 6. Adjusted p values (P<0.05=*, P<0.01=**, P<0.001=***, P<0.0001=****) are stated within figure legends and all means are displayed ± SEM. For qRT-PCR analysis, three biological samples were used for each condition. For FACS marker analysis, four independent samples were analyzed. At least 10^5 cells were counted for FACS cell populations. For the histology experiments, at least 10 biological samples were used (each animal provides 2 biological samples).

Results

Part 1. Macrophage gene expression profile during physiological sterile inflammation and tissue repair.

Cardiotoxin induced skeletal muscle damage is a robust model of immune cell assisted regeneration.

We sought to understand the role and contribution of myeloid cells, primarily macrophages and also their interactions during skeletal muscle regeneration. We chose the CTX induced skeletal muscle injury model since this model proved to be instrumental for the identification of exogenous mechanisms of muscle regeneration but it is also suitable to study the effect of the regenerating muscle upon myeloid cell activity. The model works as follows: by injecting cardiotoxin (snake venom peptide that acts as protein kinase C-specific inhibitor) intramuscularly, we induced homogenous skeletal muscle damage in the *Tibialis anterior* (TA) muscles. By day 8 after the injury the overall architecture of the muscle is restored although most regenerated myofibers are smaller and display central myonuclei, a known hallmark of recent muscle regeneration. Thus, any change in the cellular composition or altered histological features at day 8 post injury, indicates perturbations in intracellular communications.

Upon CTX injury a large number of circulating immune cells infiltrate and accumulate in a rapid and highly ordered manner inside the injured tissue. This pool of invading cells consists primarily of neutrophils, monocytes and macrophages. More specifically, after acute injury Ly6C^{high} F4/80^{low} monocytes infiltrate early at day 1 to promote the clearance of necrotic debris, whereas Ly6C^{low} F4/80^{high} macrophages emerge later through a phenotypic shift starting at day 2 and completed by day 4, to assist tissue healing. The Ly6C^{high} and Ly6C^{low} macrophages in regenerating muscle display distinct inflammatory profiles than those described in the literature *in vitro* or from the M1/M2 macrophage subtype classification and thus cannot be defined as canonical M1 or M2 macrophages. These findings support the notion that surface markers for macrophage subsets vary depending on the injury model and tissue they are extracted from.

Global gene expression profile of muscle macrophages

To extent our gene expression profiles of the muscle MFs, we performed RNA-seq analysis on the selected populations that correspond to the two main phases of inflammation in regenerating muscle; the pro-inflammatory phase (day 1-2), followed by the resolving/regenerative phase (day 4). By comparing the gene expression profiles between the macrophage subsets at each time point, we identified a series of novel molecules secreted by macrophages that could be involved in myogenesis. In addition, gene expression data showed good overlap with anticipated expression patterns of known regulators and effectors of muscle regeneration. These results indicated that an unbiased analysis of the gene expression patterns using RNA-seq (with higher sensitivity than microarrays) might report yet unidentified molecular mechanisms that are relevant to the acute and resolution phases of the inflammation within the injured muscle.

Macrophage Gdf15 regulates skeletal muscle regeneration by activating myoblast proliferation

Several strategies were tested to analyze the kinetics of gene expression by macrophage subsets during the time course of muscle regeneration. We found that the "vertical" comparison between Ly6C^{high} and Ly6C^{low} macrophage subsets at each time point provided the most robust results. Interestingly, many of the top-ranking genes in the list of genes that were upregulated at day 2 in the Ly6C^{high}F4/80^{low} vs Ly6C^{low}F4/80^{high} populations, was enriched with genes associated with inflammation such as Il1b, Clec4e, Ccr2, Nfkb2, Cebpb in accordance with the role of Ly6C^{high} macrophages in the mounting of the inflammatory response following injury. At the top of the list, an extracellular matrix proteoglycan named

versican was exclusively expressed in Ly6C^{high}F4/80^{low} macrophages, notably at the first stages of muscle regeneration. These results indicated that macrophages could be active contributors of ECM reorganization during the initial phase of muscle regeneration.

Similarly, many of the top-ranking genes in the list of genes that were upregulated at day 2 and day 4 in the Ly6C^{low}F4/80^{high} vs Ly6C^{high}F4/80^{low} populations were enriched with genes associated with mature macrophages and tissue repair, such as Igf1, Myo1e, CD36, Cx3cr1, Pparg, Stat1, Rxra, Cebpa. Among these genes, growth differentiation factor 15 was upregulated in Ly6C^{low}F4/80^{high} macrophages at the later stages of muscle regeneration at Day 2 and Day 4 respectively. Based on these unbiased findings, we hypothesized that Gdf15 could potentially influence the outcome of skeletal muscle regeneration by acting as an effector on the muscle tissue itself. To test this hypothesis, we used macrophage deficient (including bone marrow transplantations) knock-out strains for Gdf15. These 2 models exhibited a profound impairment in muscle regeneration and clearly indicated that that these genes critically contributed to muscle regeneration.

Since Gdf15 is a growth factor we wanted to characterize the GDF15 effector function, and thus we cultured primary myoblasts with or without recombinant GDF15. We found that addition of rGDF15 to the culture medium, significantly increased myoblast proliferation. We also examined the dynamics and composition of immune infiltration in injured muscle in WT and Gdf15 KO animals and found that more myeloid cells infiltrated the injured muscle in Gdf15 KO animals at day 2 and 4 post CTX. In addition, we detected significant differences in the subset composition of infiltrating MFs at day 2 and 4 after CTX injury. Collectively, these results show that Gdf15 deficiency affects both myeloid cells infiltration and differentiation within the regenerating muscle.

Macrophage PPARg regulates cell type specific genes in muscle infiltrating MFs and skeletal muscle regeneration

Recent reports suggested cell metabolism as a defining factor in MFs identity and functional status. When analyzing the RNA-seq expression data for metabolic regulators that could account for such a preference for metabolic genes, we found that a master regulator of metabolism, Pparg, was expressed at a high level in the Ly6C^{low} repair subset. Based on this finding, we hypothesized that MF PPARg is a metabolic sensor and regulator of skeletal muscle regeneration. By using two distinct genetic knock-out models we clearly show that PPARg activity in muscle infiltrative MFs, critically contributed to the timely resolution of inflammation and eventually to skeletal muscle regeneration.

As only few paracrine signaling pathways between MFs and tissue progenitors have been described, we decided to pursue the identification of the putative regulatory circuit that might connect muscle infiltrating MFs to myotube differentiation in a PPARg dependent manner. As PPARg is a transcription factor, we presumed that a relevant change in the gene expression in muscle MFs must shed light on the regulatory circuit that is abrogated in PPARg KO MFs. To identify PPARg dependent gene expression changes in muscle infiltrating MFs, we isolated representative populations of MFs from regenerating muscle (Day 1, 2 and 4 post CTX) from WT and PPARg MacKO animals and analyzed their gene expression profiles by microarrays. We were looking for genes that (1) were PPARg dependent in more than one MF subset, (2) coded a secreted factor and (3) whose activity might be linked to muscle differentiation based on existing data. Strikingly, there was only 1 gene, namely Growth differentiation factor 3 (Gdf3), that fit all these criteria and was consistently downregulated in all 4 investigated MF populations.

Macrophage GDF3 regulates myoblast differentiation and muscle regeneration

GDF3 belongs to the TGFβ family, whose members are secreted factors acting in a paracrine manner. Several members of the TGFβ family are known regulators of muscle regeneration, including GDF8 (also known as Myostatin). Therefore, we selected Gdf3 as the most likely PPARg dependent gene that

contributes to muscle regeneration for further analysis. In WT animals, the protein expression showed a pronounced induction, which peaked at day 4, a time when inflammation subsides and regenerative processes start to dominate within the injured muscle. In the PPARg deficient and Gdf3 KO animals this induction is lost, contributing to muscle regeneration delay. Exogenously added GDF3 rescued the regeneration deficit seen in these animals underlining the importance of MF derived GDF3 in skeletal muscle regeneration. In addition, it validated our hypothesis regarding the existence of a PPARg-GDF3 axis responsible for the regulation of tissue regeneration. To characterize GDF3's effector function, we cultured primary myoblasts with or without recombinant GDF3. We found that addition of GDF3 to the culture medium induced a robust effect of GDF3 on myotube formation suggesting that GDF3, once released from MFs within the injured/regenerating tissues, could regulate molecular pathways relevant to the fusion of differentiating muscle cells.

GDF3 restores muscle regeneration in aged mice

Skeletal muscle mass, function, and repair capacity all progressively decline with aging, restricting mobility, voluntary function, and quality of life. The PPARg-GDF3 regulatory axis described in our studies identifies a sensory-regulatory-effector mechanism, by which MFs are regulators of the skeletal muscle progenitor compartment. This axis orchestrates tissue regeneration, and let us to hypothesize that GDF3 expression might be impaired in geriatric mice (28-month-old), and a potential alteration of the immune response with aging is also a muscle regeneration disturbing factor, due to its role in the stimulation of satellite cell proliferation and differentiation. Indeed, by infusing rGDF3 intramuscularly, we restore the old muscles ability to regenerate, through proper activation of satellite cells. These results highlight that GDF3 is able to modulate muscle-resident cells, and possibly immune and muscle cell cross-talk during muscle repair in a geriatric model of impaired regeneration.

Part 2. Chromatin dynamics of macrophages during muscle regeneration

MARE binding TFs remodel the muscle related macrophage genome

During the next phase of our studies we were interested in what kind of epigenomic changes are taking place during macrophage subtype specification following acute sterile injury by CTX. We hypothesized that by using an unbiased genomic approach called "assay for transposase accessible chromatin" (ATAC-seq), we could study the chromatin structure and accessibility during the transition of inflammatory macrophages to repair type ones, and thus identify novel regulators of this process. ATAC-seq experiments revealed a dynamic and dramatically reorganized chromatin structure, affecting both inflammatory and repair related marker gene loci (Arg1, Il1b, Klf4, Cd68, Slc40a1, Il1r2, Tgfbr1, Cd80, Nfkb1, Mcl1, Il10, Igf1, Ptges). Using de novo motif analysis, we could detect and identify sequence motifs associated with the unique chromatin regions changing during the macrophage phenotypic transition, informing us about the binding sites of potential transcriptional regulators. Surprisingly regions that close showed more enrichment for PU.1 motifs, while on the other hand we found that the TRE and MARE motifs were enriched at the opening sites. MARE binding TFs have never been described or implicated in such dynamic chromatin changes and only 2 members of the MARE binding TF family are differentially expressed (BACH1 and NFE2L2), in inflammatory Ly6C^{high} vs repair Ly6C^{low} macrophages, suggesting that these two TFs are activated in macrophages as part of the wound response. In summary, our unbiased approach using ATAC-seq identified the presumed TFs who might be relevant in macrophage phenotypic transition and showed the dynamic nature of the regeneration process with regards to changes at the chromatin level.

Hmox1 is a direct target gene of Bach1, driven by a damage response specific enhancer cluster

Transcription factors can be categorized on the basis of their effect on gene expression. Transcriptional activators like NFE2L2 (Nuclear Factor, Erythroid 2 Like 2) promote gene expression and have signal-dependent activity. Transcriptional repressors like BACH1 (BTB Domain And CNC Homolog 1) inhibit gene expression driven by transcriptional activators and therefore function to oppose or shape their functional outcome. Therefore, to understand the function of transcriptional activators, it is essential to consider the transcriptional repressors that they antagonize. Moreover, as the process of cellular differentiation requires both activation of lineage-specific gene expression programs, and suppression of alternative lineage gene expression programs, it is important to consider the functions of transcriptional repressors in the context of the alternative lineage programs that they antagonize.

As an example of data integration, the MARE binding transcriptional repressor BACH1 is highly expressed and overrepresented in Ly6C^{high} inflammatory macrophages while its binding motif is unexpectedly enriched specifically in the open chromatin regions. BACH1 is a basic leucine zipper (bZIP) protein that is released from the chromatin in response to cell injury and inflammation, and as an end result a significant increase in the expression of antioxidant proteins, such as heme oxygenase 1 (HMOX1), an enzyme crucial for toxic heme clearance, is observed. Analysis of the Hmox1 locus using ATAC-seq data visualization, BACH1 chromatin immunoprecipitation (ChIP) and enhancer RNA qPCR measurements identified novel regulatory elements, showing extensively transcribed enhancer regions spanning 12kb upstream of Hmox1 transcription start site (TSS). In addition, a gene enrichment analysis on our RNA-seq data from CTX injured muscle infiltrating macrophages, revealed that iron and heme related pathways are being enriched in the Ly6C^{low} repair macrophages, further suggesting that the heme-BACH1-HMOX1 axis could have a crucial role in the inflammatory response and muscle regeneration outcome after CTX injury.

Heme-Bach1-Hmox1 axis is required for efficient muscle regeneration

Searching for the functional role of Bach1, Hmox1 and heme during muscle regeneration we used our CTX injury model and utilized *in vivo* heme treatments, Bach1 KO animals (including bone marrow transplantation to assess myeloid Bach1) and myeloid specific Hmox1 KO mice. Notably, Bach1 KO serves also as genetic model of Hmox1 overexpression, while *in vivo* heme treatments serves both as a transient BACH1 knock-down model and consequently a HMOX1 overexpression model. In all 3 experimental models muscle regeneration was severely impaired at day 8 post CTX. Intriguingly, the regeneration process in the Bach1 KO is still impaired even at later stages (day 21 and day 70 post CTX) while no developmental impairment was observed in Bach1 KO uninjured muscles suggesting that the muscle regeneration/growth impairment is only evident after an acute injury.

Heme-Bach1-Hmox1 axis controls the phenotypic transition of myeloid cells following CTX injury

Next, we asked whether the impaired muscle regeneration was caused by a defect in the cellular dynamics of the myeloid cell infiltrate during muscle regeneration. Interestingly, we did not find any difference in the numbers of invading myeloid cells (CD45^{+}) at any of timepoints we examined, thus excluding the possibility of a massively diminished myeloid cell invasion contributing to the muscle regeneration impairment we observed previously. However, this finding did not exclude the possibility of a change in the cellular composition and differentiation of the infiltrating myeloid cells. Surprisingly, in the case of Hmox1$^{fl/fl}$ LysMCre muscles, the phenotypic shift is severely delayed while, in the case of Bach1 KO and heme treated muscles, the ratio of Ly6C^{low} is higher. In summary, we observe significant differences in the ratios of inflammatory and repair macrophages, during their phenotypic transition suggesting a critical role for heme, BACH1 and HMOX1 in response to the acute sterile injury, showing that they function as a switch between inflammatory (Ly6C^{high}) to repair macrophages (Ly6C^{low}).

Muscle regeneration related genes are novel Bach1 targets and contribute to the impaired muscle repair phenotype

Evaluation of gene expression data from Bach1 KO muscle infiltrating macrophages revealed dysregulation of numerous genes (Il6, Il10, Dusp1, Slc40a1, Cebpb, Gdf3, Igf1, Pparg) involved in muscle regeneration and macrophage phenotype switching, suggesting that Bach1 potentially controls the expression of these important genes by regulating their enhancers. To validate these observations, we performed BACH1 ChIP experiments in BMDMs before and after heme treatment and demonstrated that BACH1 is bound at multiple enhancers, at various genomic locations and distances from the TSS of these genes. This suggests that BACH1 directly binds and regulates important inflammatory and repair related genes in the context of muscle regeneration (such as Igf1, Slc40a1, Il6, Il10, Gdf3, Pparg, Dusp1, Cebpb), which further explains the severity of the Bach1 KO delayed muscle regeneration phenotype. In summary, we show that Bach1 is an acute damage signal dependent TF having a much larger gene regulation repertoire that expands beyond iron and heme related pathways.

Part 3. Reciprocal satellite-myeloid cell communication during regeneration.

Aside from the transcriptional regulation of muscle infiltrating macrophages that we studied so far, we wanted to identify what are the cell interactions within the injured tissue cell milieu that contribute or potentially control the macrophage phenotypic switch. Here, by using the cardiotoxin (CTX) induced skeletal muscle injury combined with (1) irradiation as a means to alter cellular composition prior to injury, (2) bone marrow transplantation to replenish myeloid cells, (3) *in vivo* imaging to monitor the myeloid cell phenotypic switch *in situ*, and (4) focusing on *in vivo* ablation of a specific muscle cell population (paired box 7 (PAX7) positive cells) as a proof of concept, we tried to show that muscle tissue integrity and cell composition prior to the injury is required for *in situ* macrophage phenotypic shift and subsequently for proper and complete tissue regeneration.

The satellite cell pool is radiation sensitive and is required for proper muscle regeneration.

We first sought to validate whether muscle regeneration is indeed impaired in irradiated muscles, and second whether this effect is due to diminished satellite cell populations. Indeed, muscle regeneration in the irradiated muscles is severely impaired, even after 2 months post irradiation, in comparison to control non-irradiated or radioprotected muscles. To assess the effect of irradiation on the local muscle cell pool and more specifically on satellite cell pool, we measured the mRNA expression of a commonly used marker; Pax7 via qPCR and validated PAX7 expression through immunohistochemistry. Our data show that indeed Pax7 mRNA expression is decreased in the irradiated muscles compared to controls and radioprotected muscles at 8 weeks following irradiation. Interestingly, this effect is evident in both injured (Day 8 post CTX) and uninjured irradiated muscles (Day 0). Furthermore, PAX7 staining revealed fewer $PAX7^{+}$ cells in the irradiated muscles both at day 0 and day 8 post CTX injury. Radioprotected muscles served as control and showed similar numbers of $PAX7^{+}$ cells to non-irradiated control muscles. These results suggest that $PAX7^{+}$ satellite cells are indeed radiosensitive and are likely to be the major cause of the muscle regeneration deficiency observed in irradiated muscles.

Delayed phenotypic transition of myeloid cells in irradiated muscles following CTX injury

Next, we asked whether the impaired muscle integrity, and the altered cellular composition induced by irradiation prior to an acute injury, would have any effect on the cellular dynamics of the myeloid cell

infiltrate during muscle regeneration (even after 2 months post irradiation). Interestingly, we found a statistically significant increase in the numbers of invading myeloid cells ($CD45^+$) in the irradiated versus radioprotected muscles at both timepoints thus excluding the possibility of a massively diminished myeloid cell invasion contributing to the muscle regeneration impairment we observed. However, this finding did not exclude the possibility of a change in the cellular composition and differentiation of the infiltrating myeloid cells. Surprisingly, in the case of irradiated muscles, the phenotypic transition starts later and is completed only by day 6 (normal phenotypic shift dynamics are completed by day 4). In summary, we observe a remarkably long 2-day delay (in the context of an 8-day regeneration process) in the phenotypic transition of infiltrating myeloid cells in the irradiated muscles. In addition, it also showed that radioprotection (in the form of lead shielding) during irradiation exposure prevented the delay of muscle regeneration following CTX injury, by preserving the local muscle cell composition and by maintaining the infiltrating myeloid cell kinetics. However, we reached the limit of this method because we are unable to differentiate between the BM-derived and the tissue resident macrophages as the source of the infiltration.

Bone marrow transplantation allows establishing the source of myeloid cell invasion and phenotypic shift

In order to control the source of the infiltrating cells (BM-derived), and to exclude the possibility that the restoration of the infiltration dynamics in the radioprotected muscles is due to the protection of the resident tissue macrophages, we performed bone marrow transplantation (BMT). This method allowed us to investigate whether the infiltrating myeloid cells in both the radioprotected and irradiated muscles share the same features, and originate from the donor C57BL/6J bone marrow (CD45.2 positive). It is important to note that total body irradiation precedes BMT thus giving us the unique opportunity to combine irradiation induced cell ablation and myeloid cell tracing. In agreement with our previous lower body irradiation experiments, muscle regeneration in these BMT animals is severely impaired, even after 2 months post irradiation and BMT, in comparison to control non-BMT animals. In addition, when we further followed up the regeneration period in the BMT animals we observed that recovery to a morphologically and histochemically mature muscle takes at least 2 more weeks (Day 20 post CTX) compared to the non-BMT. Furthermore, we did not find any statistically significant differences between the numbers of invading myeloid cells ($CD45^+$) in BMT versus non-BMT controls validating our previous results. Examining the dynamics of the infiltrating myeloid cell populations during the course of the regeneration in non-BMT controls and BMT animals, we consistently observe a 2-day delay in the phenotypic transition of the infiltrating myeloid cells from inflammatory to the repair phenotype. These experiments reported to us, as expected, that irradiation is impacting regeneration in the bone marrow transplantation model as well, while we can readily assess the origin of the myeloid infiltration.

Local radioprotection during BMT allows the assessment of donor bone marrow derived invading macrophages in an intact muscle tissue environment

The next obvious question was to determine whether radioprotection (lead shielding) in the BMT-shielded animals sustains the cellular dynamics of the myeloid cell invasion following CTX injury at control levels. Indeed, radioprotection by lead shielding effectively attenuated radiation effects and protected muscle tissue integrity. In addition, to prove that the infiltrating cells in both the BMT-shielded and BMT-unshielded muscles originate from the donor C57BL/6J bone marrow (CD45.2 positive) and share the same features, we quantified $CD45^+$ positive cells according to the myeloid lineage markers CD45.1 (recipient BoyJ bone marrow stains positive for CD45.1 but not CD45.2) and CD45.2 by FACS. Over 90% of the infiltrating cells in both the BMT-shielded and BMT-unshielded muscles were CD45.2 positive which is in line with the level of chimerism detected in the blood from the same animals, thus excluding the contributions of tissue resident macrophages in the observed phenotype. By demonstrating that the donor-

derived cells infiltrate (CD45.1 vs. CD45.2 %) is present, even when the host limb is radioprotected during BMT; suggest that this BMT modification could be a viable model for muscle regeneration studies despite the tibia and femur not being irradiated. Taken together, these results suggest that the cause of the delay in regeneration is the effect of irradiation of the leg itself and not elsewhere in the body for example in the bone marrow, blood or spleen that are sites where myeloid cells could be derived from.

^{18}F-FDG PET-MRI allows *in vivo* monitoring of the infiltrating immune response and reveals that the invading myeloid cells are metabolically distinct

The analysis of myeloid cells following the injury based on surface markers requires long (over 6 hours) and tedious tissue processing. In order to obtain additional and truly *in vivo* non-invasive reading of myeloid cell invasion we turned to *in vivo* imaging. Positron Emission Tomography (PET) imaging technique is a widely used noninvasive method in clinical and preclinical studies for the detection of tumors, staging and monitoring the effect of cancer therapy. Glucose analogue, 2-[^{18}F] fluoro-2-deoxy-d-glucose (^{18}F-FDG) is the most commonly used and validated tracer, which is up taken by activated cells where it is metabolically trapped and accumulates in proportion to intracellular demands. Increased FDG uptake was observed in several energy-intensive processes, such as infections and inflammation and has been associated with activated neutrophils and macrophages, so we hypothesized that using FDG as a tracer and combining it with MRI, could be a novel and effective method to monitor the events following the acute muscle injury and specifically the metabolism of the immune cells during the inflammatory response. The ^{18}F-FDG accumulation was overall significantly higher in the injured TA muscle area in all the timepoints we checked, compared to control uninjured animals. We next asked whether the signal comes from the muscle resident cells or the infiltrating immune cells. To tackle this question, we decided to perform an *ex vivo* ^{18}F-FDG uptake on isolated myeloid CD45$^+$ versus non-myeloid CD45$^-$ cells from D1 and D2 injured muscles. In accordance with our hypothesis myeloid CD45$^+$ cells seem to uptake and utilize more ^{18}F-FDG compared to non-myeloid CD45$^-$ cells, which is line with previous observations, where macrophages co-localize with the *in vivo* PET signal in inflamed tissues.

In summary, we demonstrated that ^{18}F-FDG PET-MRI is a valuable tool to monitor immune cell invasion *in vivo*. Thus, we used it in order to delineate and further explain the difference in the regeneration process between BMT-shielded and BMT-unshielded muscles. More specifically we detected a significantly lower accumulation of ^{18}F-FDG in the BMT-unshielded muscles compared to the BMT-shielded muscles of the same animal at Day 2, Day 4, Day 5 and Day 6 post CTX.

***In vivo* ablation of Pax7$^+$ cells produces similar phenotypic transition delay on infiltrating macrophages.**

The radioprotection and *in vivo* imaging experiments presented in this dissertation suggested that the infiltrating immune response of skeletal muscle regeneration is regulated by a local radiosensitive tissue compartment, including PAX7$^+$ satellite cells. In order to determine if the decrease in satellite cell numbers observed following radiation is contributing to delayed macrophage phenotypic shift, we decided to deplete satellite cells *in vivo* before the injury. By using a transgenic mouse model for selective diphtheria toxin-induced depletion of PAX7$^+$ satellite cells we observed that satellite cell ablation (Pax7 Cre^{ER}-DTA), led to alterations not only in muscle macrophage infiltration numbers both at Day 4 and Day 6 post CTX but also in dynamic MFs phenotype shift, similar to irradiated and BMT-unshielded muscles. Therefore, our data suggest that it is indeed Pax7-lineage cell pool, which is regulating, at least in part, the repair macrophage phenotype transition. Taken together, our data reveal the existence of a reciprocal cross talk between the skeletal muscle tissue and myeloid cells recruited following the injury and establish an experimental paradigm suitable to dissect the components of immune cell invasion assisted tissue repair.

Discussion

Inflammation triggers a broad array of physiological and pathological processes and was evolved as a response to restore homeostasis. Although the pathological features of many types of inflammation are well understood, their physiological functions are mostly unknown. Thus, in order to develop specific therapeutic interventions of tissue malfunction or homeostatic imbalance (caused by dysregulated inflammation), the origins and physiological features of an inflammatory response need to be understood in a broader physiological context. The cardiotoxin (CTX) induced skeletal muscle injury model is an ideal experimental system to study physiological inflammation dynamics and tissue environment dependent monocyte-to-effector macrophage transition processes. This model induces a synchronous inflammation process allowing the isolation of homogenous cell populations. Upon injury, circulating monocytes enter the damaged tissue area and differentiate to macrophages with an inflammatory phenotype (Ly6C^{high}) and that very fast (within 1-2 days) the same cells shift to a Ly6C^{low} repair macrophage phenotype (Varga, Mounier et al. 2013). The present work reveals unexpected highly dynamic changes of gene expression by macrophage populations during acute inflammation and subsequent tissue repair, confirming the highly versatile nature of these cells that can hardly be addressed *in vitro*. We show that macrophages from regenerating muscle are distinct from resting macrophages in other tissues, while they still are true macrophages. Unbiased analysis of gene expression by macrophage populations during muscle regeneration revealed that time segregation, much more then Ly6C status segregation showed the highest differential gene expression. Thus, the time course of inflammation is the predominant organizing force in gene expression by macrophages, with an important time point at day 2-4 post injury, which corresponds to the full skewing of macrophages from Ly6C^{high} to Ly6C^{low} status and the starting of resolution of inflammation.

Several groups have independently showed that Ly6C^{high} macrophages express higher levels of pro-inflammatory cytokines (IL-1β, TNFα) and lower levels of anti-inflammatory cytokines (TGFβ, IL-10) than Ly6C^{low} macrophages. Therefore, these macrophage subsets were identified as M1 and M2 macrophages, respectively, to parallel the expression of these cytokines by *in vitro* LPS/IFNγ and IL-4 activated macrophages, respectively. The data presented here show that although Ly6C^{high} macrophages preferentially expressed pro-inflammatory markers and Ly6C^{low} macrophages preferentially expressed anti-inflammatory markers, the differences are not large, despite the limitation of global RNA analysis and the fact that low change in mRNA expression may lead to big changes in function at the protein level. These results indicate that *in vivo*, macrophages cannot be restricted and defined according to the M1/M2 nomenclature and display highly different inflammatory profiles than those previously described *in vitro*.

Comparison of Ly6C^{high} versus Ly6C^{low} macrophages, as well as comparison of the same cell subset with time revealed high dynamic changes of gene expression by macrophage subsets during muscle regeneration. These dynamics allowed to highlight 3 main features of macrophages during muscle regeneration. The first feature is early expression of acute phase proteins and scavenger receptors by Ly6C^{high} and Ly6C^{low} macrophages, respectively. These macrophages were the most numerous at day 2 and they highly expressed a series of genes involved in inflammation. Scavenger receptors are thought to participate in the removal of necrotic debris in the living body by extensive ligand specificity and a variety of receptor molecules. Here we show that Stabilin-1 is upregulated in the Ly6C^{low} fraction and its absence significantly impacts muscle regeneration. Its molecular mechanism and mode of action needs further investigation. Second, expression of ECM components during the early phase of the regeneration process. Data presented here show that Ly6C^{high} macrophages expressed ECM proteins at day 1 of regeneration. These ECM proteins are usually involved in cell-cell interactions, intercellular communication processes and regulation of ECM organization. Among them, Versican seems to be involved and differentially regulated in skeletal muscle regeneration. While the majority of such molecules have been shown to exert some effects on macrophages; mainly through chemotactic activity; their expression by macrophages themselves has

been less documented. By being exclusively expressed on day 1 Ly6C^{high} MFs it implies that it might be involved in the phenotypic transition of macrophages. In addition, our results suggest that macrophages may directly act on the ECM environment by participating to the synthesis of ECM components and molecules that regulate ECM constituents. Further investigation would decipher the significance of such an expression beside that of interstitial cells at the early phases of tissue healing. The third feature is the metabolic change of macrophages at the time of resolution of inflammation. Between day 2 and day 4 of regeneration, Ly6C^{high} macrophages undergo a switch of their phenotype, to acquire an anti-inflammatory phenotype and become Ly6C^{low} cells. Apart from the genes directly linked to the inflammatory process (chemokines, cytokines, etc.), genes involved in the regulation of cell metabolism have been shown to vary according to the inflammatory status of macrophages. However, while metabolism is foreseen as an important regulator of macrophage function, notably in diseases, few studies addressed specific metabolic pathways linked to a specific macrophage inflammatory status. For example, GDF15 has been shown to prevent obesity by increasing thermogenesis, lipolysis and oxidative metabolism. Here we identified GDF15 been differentially upregulated during the repair process in the Ly6C^{low} MFs and its absence negatively impacts muscle regeneration. A potential mechanism by which GDF15 exerts its action is by directly acting on satellite cells controlling their proliferation and expansion following the injury. In addition, we observed a strong upregulation of PPARg, which is characteristic of M2 (IL-4) macrophages *in vitro*, at the late steps of muscle regeneration (day 4) in Ly6C^{low} macrophages.

In this dissertation, we have identified the ligand activated transcription factor, PPARg as a regulator of macrophage functions during muscle regeneration. By using two distinct genetic models (conditional macrophage specific KO and BMT), it allowed us to focus on the role of PPARg in MFs. The delay in regeneration in PPARg deficient animals was profound and detectable as long as three weeks after the initial injury, thus appearing to be among the most dramatic reported deficiencies in regeneration caused by impairments in MF functions. It is interesting to note that several distinct features of the regeneration delay, such as impairment in CSA, and persistent presence of necrotic fibers were present after CTX injury in PPARg MacKO animals. Systematic transcriptomic analyses, provided clues about both the sensory and the regulatory roles of PPARg in muscle infiltrating MFs. We here described several potential new PPARg target genes. It is important to stress that earlier descriptions of direct PPARg target genes often reported lipid metabolic genes as the main targets PPARg of in MFs, which could poorly explain the anti-inflammatory role of the receptor. We report here that the transcriptional activity of PPARg is unique in muscle macrophages, because the most robustly changing genes (such as Saa3, Hebp1) are linked to inflammation, rather than to lipid metabolism. The most intriguing interpretation of the available data would be the involvement of a yet unidentified endogenous ligand for PPARg whose activity is restricted to the Ly6C^{low} compartment. Importantly, PPARg activity in the Ly6C^{low} MF subtype, which dominates the regenerative phase of muscle injury suggests that PPARg is a licensing factor for repair MFs. From the perspective of muscle regeneration, the most important finding was the identification of GDF3, which showed consistent regulation by PPARg in all relevant MF subtypes. Importantly, GDF3 is a strong candidate as a MF derived paracrine factor with muscle regenerative functions, whose diminished macrophage-linked expression is consistent with the impaired regeneration seen in PPARg deficient animals. To link macrophage biology to tissue regeneration, we analyzed the role of MF derived GDF3 in muscle regeneration in a combination of *in vivo* and *in vitro* approaches. Foremost, two genetic models of GDF3 deficiency (full body KO and BMT) reported a delay in regeneration. While the decrease in average CSA in Gdf3 KO animals was comparable to that seen in PPARg MacKO animals, Gdf3 KO animals did not display persistent inflammation and delayed clearance of necrotic fibers. This suggested that PPARg regulates several other pathways during regeneration. Importantly, a gain of function experiment revealed that exogenous GDF3 administered locally *in vivo* could counteract the deleterious effect of PPARg deficiency in MFs. In addition, GDF3 appeared to be an especially robust enhancer of myoblast differentiation and fusion. Taken together, GDF3, which is expressed in and secreted by muscle infiltrating MFs within injured and regenerating muscles, has the capacity to elicit biologically

relevant responses in primary myoblasts and differentiating myotubes and is a regulator of both *in vitro* muscle differentiation and muscle regeneration *in vivo*. As other cell types are also involved in the regeneration process, it cannot be excluded that other TGFβ family members such as GDF15 are active during regeneration and that it has effects on other cell types such as fibro/adipogenic progenitors as well.

Our findings also carry potential implications for pathological circumstances in which muscle damage and asynchrony in repair due to aging leads to severe impairment in muscle function. Here, we show that GDF3 seems to be a regulator of muscle regeneration in geriatric models as well, which are most of the time associated with the permanent presence of inflammatory cells. These findings suggest that MF derived factors such as GDF3 and GDF15 could also be used as part of other therapeutics to regulate not only regeneration, but also muscle and bone growth. To conclude the present work, so far, highlights an unexpected dynamic of the molecular signature of macrophage subsets during skeletal muscle regeneration and the identified PPARg is a required metabolic sensor and transcriptional regulator of repair macrophages, that controls the expression of GDF3, which in turn regulates the restoration of skeletal muscle integrity by promoting muscle progenitor cell fusion (Varga, Mounier et al. 2016). However, our understanding is fairly limited regarding the role of other transcription factors sensing the macrophage's tissue environment especially in the context of epigenetic and transcriptional regulation as well as contributing to macrophage effector functions. A logical extension to these studies was to employ unbiased epigenomic profiling, to identify molecules regulating phenotypic transition and effector functions of inflammatory and repair macrophages.

The phenotypic transition from the monocyte progenitors to inflammatory Ly6C^{high} and then to repair Ly6C^{low} cells is highly correlated with the tissue regeneration kinetics and is accompanied by a dynamic crosstalk between macrophages and other muscle tissue components that lead to a hierarchical chromatin and transcriptional reprogramming process. In order to delineate the order of transcriptional events during monocyte infiltration and *in situ* macrophage differentiation and to identify novel candidate genes and signaling pathways regulating *in situ* macrophage function and tissue regeneration, we sought first to map these changes by generating chromatin accessibility maps (genomic regions that are highly bound by transcription complexes) using the ATAC-seq method. By using ATAC-seq on circulating monocytes and muscle infiltrating macrophages from day 1, 2 and 4, detection of differentially opening sites in time allowed us to expand our analysis and look for the actual transcriptional regulators that might be responsible for these changes. Coupling standard bioinformatic analysis with innovative clustering algorithms, we found that a large class of more than 9000 genomic regulatory elements is becoming de novo accessible during monocyte infiltration in the muscle. Importantly, motif analysis showed that these sites were very highly enriched (58% of sites) to AP-1 motif (including the Maf recognition element - MARE) compared to other common macrophage specific motifs (PU.1, C/EBP). This intriguing finding prompted us to ask the following questions: 1) what are the AP-1 motif binding transcription factors that potentially regulate these de novo accessible sites, and 2) how these AP-1 factors contribute overall to macrophage polarization during muscle regeneration. Potential candidates would include known AP-1 factors such as Jun, Fos, NF-E2 and others that have been shown to form homo- or hetero- dimers to activate their gene targets. However, such factors are usually inducible and bind to DNA by continuously competing with other AP-1 family members. This mode of action is interesting as it suggests that repressor factors bind to low accessible sites and precede activators and that such early chromatin priming by bookmarking transcription factors might be important to activator recruitment and thus to the effective control of transient activation programs. We hypothesized that such transcription factors are in place in our experimental system and we set the following criteria to narrow down candidate regulator transcription factors: 1) ability to bind AP-1 motifs with repressor activity, 2) high expression in monocytes and macrophage populations and 3) direct sensor of cellular environment changes (such as metabolite concentration changes). It has been shown that during muscle damage, profound changes in the muscle expression of heme and iron gene can be observed. Muscle-infiltrating macrophages carry out this function thus preventing the deleterious effects caused by its

oxidizing action. In addition, the ability of infiltrating macrophages to export iron is crucial to avoid fat accumulation during the regeneration process. Myoglobin, which is released because of the muscle necrosis possibly influences the expression of iron homeostatic genes which in turn might reflect on macrophage phenotype switch. Thus, based both on literature search and available transcriptomic data, we narrowed down to the heme sensing transcription factor BACH1.

The transcription factor BACH1 forms heterodimers with small Maf proteins that binds to the Maf recognition element (MARE), a subcategory of AP-1 motif, to act as transcription repressor. BACH1 is expressed in most cell types, but it is more highly upregulated in hematopoietic cells (macrophages, dendritic cells and thymic T cells). Notably, BACH1 possesses a heme-binding region and thus can be directly bound by free heme that is released by many hemoproteins such as hemoglobin and myoglobin usually following acute tissue damage. Heme binding destabilizes BACH1, thus inhibiting its DNA binding capacity, and subsequently BACH1 is exported from nucleus and degraded in the proteasome. Subsequently, the transcription factor NF-E2, competing for the same MARE sites, heterodimerizes with small Mafs and activates BACH1 target genes, such as the heme oxygenase 1 enzyme (Hmox1) to further degrade heme to less harmful metabolites, biliverdin, ferrous iron, and carbon monoxide. Absence of BACH1 leads to constitutive expression of Hmox1. Thus, according to existing literature, BACH1 functions as a metabolite driven regulator of heme and iron metabolism, metal detoxification and cellular signaling programs. BACH1 has largely an invariable gene expression profile among different populations of macrophages and, interestingly, it has been predicted by transcriptomic analysis of the Immunological Genome Project (Immgen) to be among the core regulator of macrophage identity, along with TCEF3, C/EBP-α, and CREG-1. However, contrary to expectations based on its importance and ubiquitous presence, the existing mouse model has minimal macrophage developmental defect and no overall physiological defects in mouse development and lifespan. In addition, in experimental injury and inflammatory models, these animals show protective phenotypes and dampened overall inflammatory responses. To test our hypothesis that the heme-BACH1-Hmox1 axis contributes to macrophage function during muscle injury and regeneration, we performed CTX experiments in heme treated, Bach1 KO and Hmox1 conditional KO mice. Histology and morphometric analysis showed that these mice have delayed muscle repair and regeneration kinetics (extensive and persistent necrotic content in the muscle tissue and smaller mean muscle fiber size compared to controls). Bone marrow transplantation experiments showed that this defect is intrinsic to myeloid cells and not attributed to BACH1 function in muscle cells. FACS analysis showed comparable cell numbers of infiltrating myeloid cells but a reproducible higher (in the case of Bach1 KO and heme treated mice) or lower (in the case of the macrophage conditional Hmox1 KO) ratio of repair (Ly6C^{low}) to inflammatory (Ly6C^{high}) macrophages at day 4 post CTX. To our knowledge only three other experimental systems (DUSP1, AMPKa1 or IGF1 deficiency in muscle infiltrative MFs) were reported to lead to altered MF differentiation.

Complementary to this *in vivo* analysis and to gain insights to the molecular function of BACH1, we used eRNA measurements, and the BMDM differentiation system to map BACH1 novel binding sites and potential target genes using ChIP. We found that BACH1 binds to a large number of activated enhancers and accessible sites in the genome based on the ATAC-seq mapping. Importantly, BACH1 extensively binds to a number of gene loci (Il6, Il10, Cebpb, Dusp1, PPARg, Igf1, Slc40a1, GDF3) previously shown to participate and heavily impact the regeneration process. These observations expand the current view of BACH1 as mere rheostat of heme-oxygenase 1 expression and potentially suggest BACH1 involvement in a bookmarking mechanism of genomic sites yet to be activated in macrophages when appropriate signals become present or of an active repressing mode action to genomic regions, active in other cellular states, environments or lineages. Taken together, these results suggest that proper muscle regeneration depends on tightly and timely regulated inflammatory responses following acute injury and that BACH1 likely acts to fine-tune activation, phenotypic transition and resolution of inflammatory programs. Lastly, BACH1's protein structure and capacity to bind metabolites and sense the tissue micro-environment makes it an

attractive model to test how such signal integrators sense different tissue stimuli and shape different transcriptional programs and diverse macrophage subtypes.

Tissue regeneration is a complex but precisely coordinated array of processes involving cellular recruitment, differentiation, phenotype switches, and intercellular communications. It is remarkable, though, that the key elements of the myogenic cross talk between macrophages and myoblasts suggest that these two cell types and their interactions are critical to support regeneration. In the final part of this work, we document that (1) altered cellular composition affects the *in situ* phenotypic transition of invading myeloid cells to repair macrophages, thus (2) revealing the existence of reciprocal intercellular communication between satellite cells and recruited macrophages during skeletal muscle regeneration, and (3) establish a combination of methods to use bone marrow transplantation, with local muscle radiation shielding, to identify myeloid cell contribution to tissue repair and monitor the myeloid cells metabolic activity *in vivo.*

As mentioned several times in this work the skeletal muscle microenvironment is very heterogeneous, with distinct cell types such as satellite cells, fibroblasts, fibro/adipogenic progenitors (FAPs) adipocytes, epithelial cells, Schwann cells, and blood cells, all of which may influence and, in turn, be influenced by local structural and biochemical cues. The general consensus holds that satellite cells are required for muscle regeneration. Here we show that both, radiation-induced or *in vivo* ablation (PAX7-DTA) of these cells, leads to delayed repair macrophage phenotypic transition and muscle regeneration. While there have been several papers demonstrating that infiltrating macrophages influence satellite cell behavior, our data indicate that the reverse is also true, and that Pax7-lineage cells are regulating a degree of the *in-situ* macrophage phenotypic shift. However, there are also additional cell types that are abundant in healthy muscle, and it's been recently shown that FAPs, fibroblasts, endothelial cells, and other interstitial cells like pericytes, expand following acute muscle damage, to transiently establish an environment that enhances myogenic differentiation. This raises the question whether perturbations of their cellular interplay with satellite cells or macrophages could further explain the delay in the macrophage phenotype switch. For example, it is known that angiogenesis is crucial for muscle repair and satellite cell survival. In this context, endothelial cells strongly stimulate myogenic cell growth while myogenic cells promote angiogenesis. On the other hand, during return to homeostasis, periendothelial cells facilitate the return to quiescence of a subset of muscle precursor cells that ensures self-renewal of the satellite cell pool. Interestingly, depletion of $PAX7^+$ cells *in vivo* has no impact on vasculature ($CD31^+$ endothelial cells), innervation or neuromuscular junctions. The same group also documented that FAPs and fibroblasts are unchanged in the $PAX7^+$ ablated muscles, which suggests that the interaction of satellite cells with macrophages is unique and likely to be specific.

Our findings that PAX7 positive satellite cells affect macrophage phenotypic transition poses several more questions, regarding a potential niche for macrophages formed by satellite cells, what signaling molecules/cytokines influence the communication between these cell types and whether satellite cells produce signals that instruct macrophage functions such debris clearance, phagocytosis and myogenesis. It will require selective depletion or replenishment of satellite cells and other cell types to establish the cause and effect relationships between them and macrophage invasion, phenotypic switching and contribution to repair. Future studies should test whether radiation causes a depletion of these and other local interstitial cells with myogenic potential that could affect also the cross talk with the immune cells following acute damage, which in turn can affect macrophage infiltration and phenotypic dynamics. In this context, our data suggest that the delayed transition is likely to be causative of impaired regeneration.

Several important signaling pathways and transcriptional regulators such as IGF-1, CEBPb, AMPKa1 and P-38/MKP, have been implicated in the phenotypic transition of these muscle macrophages (from the pro-inflammatory/phagocytic phenotype ($Ly6C^{high}$) to the anti-inflammatory/reparatory phenotype ($Ly6C^{low}$)) using the CTX model. However, many more pathways could be implicated and further work towards their identification is needed. To this end, loss of function experiments, have been employed to

identify key genes, by crossing gene specific floxed allele containing mice with the myeloid-specific LysM-CRE strain, in order to generate myeloid-specific knock out strains. However, the availability of conditional myeloid specific knockout mice for most genes is a limiting factor in delineating their role in the macrophage phenotype switch during muscle injury and regeneration. A commonly used method to assess the contribution of myeloid cell populations in *in vivo* murine disease models is bone marrow transplantation (BMT). During this CD45 congenic method, the bone marrow of animals is eliminated by total body irradiation and then reconstituted by injecting a fresh bone marrow isolated from specific donor animals that are usually genetically manipulated. The fact that the congenic donor cells can be easily tracked is also highly beneficial. However, the requirement for total body irradiation leads to unwanted inflammation, tissue injuries and fibrosis if not properly optimized. Therefore, we reasoned that the combination of an optimized BMT method with dose fractionation and *in vivo* imaging would allow us to trace and characterize myeloid cell invasion and cellular interactions and overcome the radiation effect. Low radiation doses lead to incomplete engraftment due to survival of the host bone marrow, but at the same time can also lead to macrophage activation. Thus, an optimum radiation dose is needed to achieve complete BM ablation without causing any local organ damage. According to the current literature irradiation doses of 7 to 13 Gy are myeloablative but one needs to be aware that the higher the dose, the higher the risk for the animals to die from irradiation-induced toxicity. As mentioned above methods for reducing radiation toxicity include dose fractionation (in which smaller doses of radiation are administered sequentially) and lead shielding. The latter method was successfully used by Labar et al and Murphy et al. to protect the lungs and study alveolar macrophage turnover following BMT, but were limited by high levels of mixed chimerism in the circulation due to head and forelimbs being lead shielded as well. In our study and in order to bypass these limitations, (1) we used radioprotection sparingly, focused on protecting only one hindlimb (to minimize radioprotection of the recipient animal's BM), (2) increase the number of transplanted cells to 20×10^6 cells/BMT animal in order to increase the degree of chimerism in favor of donor BM repopulation (hindlimb radioprotection increases survival of host BM that in turn increases the competition with the donor BM), and (3) increase recovery time to 12 weeks post BMT, thus allowing us, both to reach high levels of blood chimerism (>90%) and protect the local radiosensitive muscle compartments (e.g. satellite cell pool).

Our combined method with *in vivo* tracing of myeloid cells by PET-MRI is an important advance allowing real time assessment of cell invasion as opposed to cell sorting that requires several hours of processing. These findings have general ramifications because it may be possible to extend the utility of this system to other organs, for studies that require preservation of resident cell populations in bone marrow transplanted animals, and especially in cases of immune cell invasion. In addition, this method allowed us to examine, monitor and characterize the immune cell invasion following acute injury by CTX. The PET-MRI *in vivo* imaging studies showed that these invading cells seem to have special metabolic properties biased towards glycolysis. Our findings suggest that ^{18}F-FDG PET-MRI is a useful tool to monitor immune cell invasion and to help to delineate and further explain the difference in the regeneration process in the irradiated muscles and especially the difference between BMT-shielded and BMT-unshielded muscles. Further acute sterile injury models in other tissues, such as liver or skin injury, could validate our PET-MRI results.

In conclusion, our work has translational implications on the muscle regenerative potential of the elderly following radiation therapy. Our findings suggest that irradiation of skeletal muscle might impair the ability of satellite cells to orchestrate macrophage phenotypic transition further impacting regeneration potential. This is in addition to other known side effects of irradiation such as direct effects on myoblasts. Our findings also convey potential ramifications for pathological circumstances in which recurrent muscle damage and satellite cell mediated regeneration asynchrony leads to devastating muscle diseases, such as Duchenne Muscular Dystrophy, which are usually associated with the permanent presence (and potential interaction) of inflammatory cells, especially macrophages.

Summary

Skeletal muscle regeneration is a complex interplay between various cell types including invading macrophages. Their recruitment to damaged tissues upon acute sterile injuries is necessary for necrotic debris clearance and for coordination of tissue regeneration. This highly dynamic process is characterized by an *in situ* transition of infiltrating monocytes from an inflammatory (Ly6C^{high}) to a repair (Ly6C^{low}) macrophage phenotype. System-level gene expression analysis revealed that the time course of muscle regeneration, much more than Ly6C status, was correlated with the largest differential gene expression. This indicated that the time course of inflammation was the predominant driving force of macrophage gene expression. These findings validate the dynamic nature of the macrophage response and associate a specific gene signature to predictive specialized functions of macrophages at each step of muscle regeneration. However, the gene regulatory events supporting the sensory and effector functions of macrophages involved in tissue repair are not well understood. Here, we show that the lipid activated transcription factor (TF), PPARg is required for proper skeletal muscle regeneration acting in repair MFs. PPARg controls the expression of the TGFβ family member, GDF3, which in turn regulates the restoration of skeletal muscle integrity by stimulating myoblast cell fusion. In addition, to delineate the order of transcriptional events during monocyte infiltration and *in situ* macrophage differentiation we generated chromatin accessibility maps using ATAC-seq. We found that a large class of genomic regulatory elements is becoming de novo accessible during monocyte infiltration in the muscle, and motif analysis showed that these sites are highly enriched to the MARE motif, compared to other common macrophage specific motifs. We also identified BACH1, a heme-regulated MARE-binding TF, as a novel regulatory molecule. The contribution of this molecule and downstream targets such as Hmox1, have been evaluated using full body and macrophage-specific knock-outs. Surprisingly, the inactivation of either Bach1 or Hmox1 in macrophages impairs muscle regeneration by altering the dynamics of the macrophage phenotypic transition. In addition, Bach1 deletion leads to transcriptional deregulation of critical inflammatory genes in macrophages upon injury. By using bone marrow-derived macrophages, we found BACH1 to bind extensively to enhancers of these genes, suggesting that fine-tuning of transient inflammatory transcriptional programs in macrophages during tissue injury, largely depend on MARE-binding TFs. Overall, this work establishes PPARg and BACH1 as required environment sensors and transcriptional regulators of muscle infiltrating MFs. Moreover, this work also establishes GDF3 as a secreted extrinsic effector protein acting on myoblasts and serving as a regeneration factor in tissue repair.

The importance of the macrophage phenotypic shift and the cell cross-talk of the local muscle tissue with the infiltrating macrophages during tissue regeneration upon injury are also not fully understood and their study lacks adequate methodology. Here, by using an acute sterile skeletal muscle injury model combined with irradiation, bone marrow transplantation and in vivo imaging we show that preserved muscle integrity and cell composition prior to the injury is necessary for repair macrophage phenotypic transition and subsequently for proper and complete tissue regeneration. Importantly, by using a model of in vivo ablation of PAX7 positive cells, we show that this radiosensitive skeletal muscle progenitor pool contributes to macrophage phenotypic transition following acute sterile muscle injury. Taken together, our data suggest the existence of a more extensive and reciprocal cross-talk between muscle tissue compartments, including satellite cells, and infiltrating myeloid cells upon tissue damage. These interactions are shaping the macrophages in-situ phenotypic shift, which is indispensable for normal muscle tissue repair dynamics.

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List of publications related to the dissertation

1. **Patsalos, A.**, Pap, A., Varga, T., Trencsényi, G., Contreras, G. A., Garai, I., Papp, Z., Dezső, B., Pintye, É., Nagy, L.: In situ macrophage phenotypic transition is affected by altered cellular composition prior to acute sterile muscle injury.
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2. Varga, T., Mounier, R., **Patsalos, A.**, Gogolák, P., Peloquin, M., Horváth, A., Pap, A., Dániel, B., Nagy, G., Pintye, É., Póliska, S., Cuvellier, S., Ben Larbi, S., Sansbury, B. E., Spite, M., Brown, C. W., Chazaud, B., Nagy, L.: Macrophage PPAR[gamma], a Lipid Activated Transcription Factor Controls the Growth Factor GDF3 and Skeletal Muscle Regeneration.
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List of other publications

3. Lyroni, K., **Patsalos, A.**, Daskalaki, M. G., Doxaki, C., Soennichsen, B., Helms, M., Liapis, I., Zacharioudaki, V., Kampranis, S. C., Tsatsanis, C.: Epigenetic and Transcriptional Regulation of IRAK-M Expression in Macrophages.
   J. Immunol. 198 (3), 1297-1307, 2017.
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List of Presentations

- 64th Congress of Biochemistry and Molecular Biology, Hellenic Society of Biochemistry and Molecular Biology, Athens, Greece, 6th - 8th December 2013.
- 2014 New Directions in Biology and Disease of Skeletal Muscle Conference, Chicago, IL, USA, June 29 - July 2, 2014.
- FEBS Workshop on Molecular Medicine and Life Science Education, Debrecen, Hungary, August 24-25, 2014.
- Danube Epigenetics conference (Conference travel grand awarded, Oral Presentation – Poster) November 19-21, 2014.
- Workshop on Ethics and Scientific Communication, University of Lausanne, Switzerland, June 26-27, 2015.
- Focus on Metaflammation Conference, University Hospital (CHUV), Lausanne, Switzerland, June 30, 2015.
- FEBS ALC Nuclear Receptor Signalling, Spetses, Greece, August 23-28, 2015 (Conference travel grand awarded, Poster was chosen for Oral presentation).
- 2016 New Directions in Biology and Disease of Skeletal Muscle Conference, Orlando, FL, USA, June 29 - July 2, 2016 (NIH travel award for outstanding poster presentation).
- 2017 Advances in Skeletal Muscle Biology in Health and Disease Conference, Gainesville, FL, USA, March 8 – March 10, 2017 (Foreign travel grand awarded and poster was chosen for oral presentation).

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