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Summary

Following skeletal muscle damage, complex cellular events are required that coordinate the restoration of the functional environment of muscle fibers. The immune system fulfills important roles in recognizing the damaged environment and mediating muscle regeneration. Two time-dependent and functionally distinct phases of muscle regeneration can be distinguished. The first pro-inflammatory response involves the expression of cytokines that mediate the early invasion of M1-phenotype macrophages after muscle damage. Within the first three days after damage, these macrophages are functionally involved in the removal of cell debris associated with the expression of cytokines that induce proliferation of satellite cells (SCs). A time-dependent change in the expression of cytokines within three to seven days after injury initiates the type 2 immune response associated with increased accumulation of regulatory T-cells. Within this time frame, the shift of macrophages to a pro-regenerative M2 phenotype occurs, associated with extracellular matrix production, inhibition of SC proliferation and the onset of differentiation. M2 macrophages further activate fibro-adipogenic precursor cells (FAP) that contribute to extracellular matrix production. The crucial switch of macrophage phenotypes is induced by the release of IL-10 and TGF-β cytokines, but also supported by the activation of AMPK. Localized IGF-1 release by macrophages essentially supports the myogenic program and subjects satellite cells to differentiation. Myotube formation, extracellular matrix production and angiogenesis finally contribute to the restoration of the skeletal muscle functional environment. Here, we give a short overview of the major cytokines, modulators and interacting cells that contribute to and coordinate immune responses to promote muscle regeneration.

Zusammenfassung

Akute Muskelverletzungen benötigen im Nachgang eine Reihe komplexer molekularer Mechanismen, die die Regeneration des verletzten Gewebes und eine umfassende funktionale Wiederherstellung aller Substrukturen regulieren. Das Immunsystem unterstützt diesen Prozess, indem es die akute Schädigung im Gewebe wahrnimmt und den notwendigen Regenerationsvorgang maßgeblich steuert. Dies geschieht durch zwei zeitlich und funktional unterschiedliche Phasen. Im ersten, pro-inflammatorischen Abschnitt induzieren Zytokine eine frühe Einwanderung von Makrophagen des Typs M1 in das verletzte Gewebe. Innerhalb der ersten drei Tage bewirken diese Makrophagen einen Abbau von zerstörten Zellstrukturen. Assoziiert mit diesem Zeitfenster ist eine Expression von Zytokinen, die die Proliferation von Satellitenzellen fördern. Eine Änderung des Zytokinprofils zwischen drei und sieben Tagen nach akuter Schädigung initiiert die Typ-2-Immunantwort, die mit einem Anstieg T-regulatorischer Zellen am Ort der Schädigung verbunden ist. Hierdurch wird eine Verschiebung der Makrophagenpopulation in Richtung des pro-regenerativen Typs M2 induziert. Diese initiieren die Produktion von Extrazellulärmatrix, hemmen die Proliferation von Satellitenzellen und initiieren die Frühphase der Differenzierung. M2-Makrophagen aktivieren zusätzlich fibro-adipogene Vorläuferzellen (FAP), die zur Matrixsynthese beitragen. Dieser essentielle Shift des Makrophagenphenotyps wird durch die zunehmende Freisetzung von IL-10 und TGF-β eingeleitet und durch die Aktivierung der AMPK unterstützt. Die durch Makrophagen initiierte, lokale Freisetzung von IGF-1 an den Ort der Schädigung finalisiert das myogene Programm und die Differenzierung der Satellitenzellen. Die hierdurch generierten Myotuben sowie erhöhte Matrixsynthese und Angiogenese gewährleistet letztendlich die Wiederherstellung des funktionalen Environments im Skelettmuskel. Wir geben hier einen kurzen Überblick über die wichtigsten Zytokine, Regulatoren und interagierenden Zelltypen, welche die Immunregulation nach akuter Verletzung im Zeitverlauf koordinieren und die Muskelregeneration steuern.

KEY WORDS:

Skeletal Muscle, Cytokines, Immune Response, Regeneration

SCHLÜSSELWÖRTER:

Skelettmuskel, Zytokine, Immunantwort, Regeneration

Introduction

Skeletal muscle is a molecular and metabolic machine that provides the basis for human movement and any form of physical exercise. Due to its major role in generating high mechanical forces, but also because of its localization at the superficial layer of the human body, skeletal muscle is prone to direct and indirect muscle damage (13, 40, 47).

The different recovery stages after skeletal muscle injury imply unique molecular and cellular regulation patterns and involve the innate immune response. The coordination between inflammation and muscle regeneration is of considerable importance in regulating a full regenerative process in skeletal muscle. The interplay of pro-and anti-inflammatory



cytokine expression in tightly controlled time frames regulates macrophage function and the disposal of damaged tissue and cell debris in the early phase after damage. In the secondary phase, type 2 immune responses coordinate the activation of mechanisms that trigger tissue renewal. This includes satellite cell activation, angiogenesis and extracellular matrix synthesis, all of which are necessary to rebuild the functional environment of skeletal muscle. Here, we provide a compact overview about the main roles of the immune system that support and regulate skeletal muscle regeneration after injury. The mechanisms are excellently reviewed in the paper of Tidball (43), which also builds the framework of this article. Figure 1 highlights the time course of important events that coordinate the immune system with muscle regeneration.

Skeletal Muscle Satellite Cells and Muscle Damage

Muscle damage takes place along a continuum from minor ultrastructural perturbations (47) to devastating and large injuries within extended areas of muscle. Its regeneration relies mainly on the disposal of damaged cell material and the coordinated synthesis of new proteins restoring the functional muscle environment. Muscle damage on the ultrastructural level can be compensated within few days (e.g. after exercise) and involves for instance localized cell membrane disruption or protein unfolding within Z-disks (47). At this level, spatially localized autophagy and protein synthesis may be sufficient to regenerate damaged proteins (23).

However, damage on a structural level is associated with ruptures of extended areas of muscle, extracellular matrix, capillaries and subsequent intramuscular bleeding (21). A full regeneration of muscle requires a program that proliferates and differentiates satellite cells (SCs), but also activates mechanisms that induce angiogenic processes and extracellular matrix synthesis (43). Satellite cells also named as myogenic precursor cells (MPCs) reside quiescent and non-proliferating in a niche between basal lamina and sarcolemma of skeletal muscle fibers (15). Upon stimulation or muscle damage, these "muscle stem cells" get activated, leave their quiescent state and proliferate in a way called asymmetric cell division. This means that one daughter cell is committed to further differentiation, while the other cell returns in the quiescent state. Based on the degree of damage and time course of stimulation, a proportion of daughter cells start to differentiate into multinucleated myotubes. From here, they enter a stage called terminal differentiation and growth, which is vital for the regenerative process. Terminally differentiated myotubes can further fuse with existing myofibres (22).

The other proportion returns to the quiescent state, where they replenish and maintain the satellite cell pool. While in healthy and young skeletal muscle the pool of SCs can be maintained, aging and substantial muscle damage reduces the amount of residing SCs and impairs the regenerative potential of SCs (5). Each step of the process is associated with specific changes in the expression of myogenic transcription factors (PAX7, MyoD, Myf5, Myogenin) which are controlled by master regulatory genes (38). While quiescent SCs express PAX7 but not MyoD, the early activation of SCs also induces MyoD expression. Mutations of MyoD or NUMB (protein numb homolog) proteins (these regulate asymmetric cell division) were shown to disturb differentiation and proliferation of SCs impairing muscle regeneration (14). The innate immune response after acute damaging events (co)-regulates the expression of these genes in a coordinated manner (see later paragraphs).

Immune Responses in Early Phases of Muscle Regeneration

Leucocytes are highly abundant in skeletal muscle with a reported number from up to 2000 leucocytes per mm³ (30). This cell population consists of various subgroups e.g. CD8⁺ cytotoxic cells, regulatory T-cells (T-reg), neutro- and eosinophile cells, which all build smaller proportions of leucocytes in muscle. The vast majority of intramuscular leucocytes are represented by macrophages and monocytes which, similar to SCs, reside in a quiescent non-activated state at the surrounding extracellular matrix of muscle fibers or in close vicinity to blood vessels (18). Their immediate activation by trauma and exercise is indispensable and significantly affects later stages of muscle regeneration.

Acute exercise, trauma, muscle-specific diseases or experimentally injected toxins cause a variable degree of early inflammatory responses associated with a significant rise of intramuscular leucocytes. Within 12-24 hours, neutrophils expressing LY6G and CD11b invade damaged muscle and reach maximum numbers (11). Macrophages induce this neutrophil influx by releasing the neutrophil chemo-attractants CXC chemokine ligand 1 and CC Chemoligand 2 (CXCL1 and CCL2) (51). CCL2-mediated signaling is of vital importance for inducing muscle inflammation and regeneration, as mutations within the genes of CCLs or its receptor (CcR2) reduces macrophage numbers and impairs muscle regeneration by attenuating muscle growth (30).

Also, T-cells are early responders to acute muscle damage and have important roles in coordinating the cascade of steps that regulate muscle repair. Signaling through the T-cell receptor of CD8⁺ T-cells significantly promotes the innate immune response which is vital for the induction of regeneration. Cd8a deletion has been shown to reduce CCL2 production by T-cells and impairs macrophage recruitment in muscle (53).

The early invasion of neutrophils is an indispensable response in the acute phase of muscle damage and affects the activity of other immune cells by preparing the inflammatory environment (46). After invasion of neutrophiles, macrophages and monocytes extravasate from the blood stream into the damaged environment and release the pro-inflammatory cytokines interferon-gamma (IFN- γ) and tumor necrosis factor (TNF). This drives the switch of macrophages to the activated pro-inflammatory phenotype M1 (M1 macrophages) (32). The nomenclature reflects the origin of activation by pro-inflammatory T helper 1 ($T_H 1$)-type cytokines and separates them from M2 macrophages that are later activated in the anti-inflammatory phase and associated with tissue repair (1).

This initial pro-inflammatory phase is significantly increased within 24 hours and extends up to 48 hours post damage (42, 43). During this time frame, the proliferation of SCs is significantly increased. In the following phase, the pro-inflammatory response is increasingly attenuated but associated with a phenotype shift from M1 to M2 macrophages and reduced proliferation of SCs. This time point corresponds with the onset of the type 2 immuno-response and is initiated by a change in cytokine profile (IL-10, IL-4) (10). M2 macrophages are activated by TH2-type cytokines and associated with the resolution of the pro-inflammatory state and finally tissue repair. They peak around 4 days after injury, but remain elevated up to 14 days post injury (43). Thus, during early and later stages of muscle regeneration, macrophages are always present as a mixture of M1 and M2 phenotypes, but with a shifting emphasis in dependency of the phase of regeneration.

Inflammatory Environment, Satellite Cell Activation and Macrophages In Early Phases Of Muscle Regeneration

Pro-inflammatory responses are linked to muscle regeneration, as they regulate initial phases of satellite cell activation, proliferation as well as early phases of differentiation. The early stages of repair and regeneration are strongly influenced and controlled by IFN- γ . It acts on myogenic cells via the class II - major histocompatibility complex, transactivator (CIITA) pathway which modulates the myogenic gene expression program (27). IFN- γ is of vital importance for these events, as elevated levels coincide with increased numbers of neutrophils, macrophages and importantly MyoD expression in SCs within the first 24h after damage (7). IFN- γ binds to its receptor on SCs (MPCs) and activates the Janus kinase pathway (JAK-STAT1), which induces expression of various target genes, also involving CIITA (34). CIITA is required for the inhibition of the differentiation process in SCs, in order to retain them in a proliferative state. This may potentially ensure a substantial increase in satellite cells within damaged areas of skeletal muscle. By adding more potential nuclei to the damaged tissue environment, the transcriptional infrastructure for further protein synthesis is improved. However, while this mechanism is necessary in the early stages of tissue regeneration, it must be shut down in later stages to enable the differentiation of SCs (43).

In this time frame, a major task is the removal of defective structures and cellular debris in skeletal muscle via phagocytosis by M1 macrophages (48).

Inhibition of IFN- γ signaling reduces the expression of genes in macrophages that indicate the M1 phenotype (e.g. inducible nitric oxide synthase; iNOS and interferon-regulatory factor 1; IRF1) (7). Recent findings have shown that altered STAT-1 signaling contributes to the IFN- γ -mediated activation of macrophages in regenerating muscles (25).

This dual role of IFN- γ , controlling SC differentiation and macrophage phenotype is crucial within a time period of one to five days after injury (7).

Immune Responses during Terminal Phases of Muscle Regeneration

Maximum numbers of phagocytic M1 macrophages appear around 48 hours after acute injury in muscle. This phenotype of macrophages is then replaced by the non-phagocytic M2 macrophages peaking between four and seven days post injury. This coincides with a change in the gene expression pattern towards a state of terminal differentiation in damaged tissue (41). This includes the necessity to inhibit the proliferation of SCs and to submit the cellular environment progressively towards states of myogenic differentiation. The regulation of this process requires a multitude of factors.

CD163 is a specific marker of macrophages, which is highly abundant during terminal differentiation of SCs in damaged muscle. It is a transmembrane glycoprotein which is expressed by macrophages, strongly dependent on the cytokine profile. While IL-10 increases its expression, TNF reduces it. CD163 binds to hemoglobin-haptoglobin complexes enabling its internalization and degradation. This is of crucial importance, as local hemolysis produces toxic levels of hemoglobin, which is able to amplify tissue damage. Moreover, CD163 promotes the expression of IL-10 hereby supporting its anti-inflammatory effects. The systemic ablation of CD163 is associated with reduced regenerative potential due to a slowed myogenic program (42). During injury, CD163 extracellular domains in the local

environment are released and deactivate the pro-inflammatory cytokine TWEAK (TNF-related weak inducer of apoptosis). TWEAK promotes MPC (SC) proliferation in muscle by activating NF κ -B signaling. CD163 mediated inhibition of TWEAK may hereby reduce the proliferation of SCs and facilitate the induction of the differentiation process in the myogenic program (4).

The time-dependent transition from the initial immune response with dominant M1 macrophage occurrence to a M2 based macrophage population is indicative of a transition towards enhanced myogenesis in muscle and necessary for a coordinated schedule of regeneration of injured skeletal muscle. Multiple factors coordinate the immune environment of skeletal muscle and couple it with different stages of myogenesis. The phagocytic removal of cellular debris after damage not only creates more cellular space, but also offers specific roles in muscle regeneration. Macrophage phagocytosis suppresses the expression of TNF and increases that of TGF- β indicating a shift from M1 to M2 macrophages (1). Consequently, the event of phagocytosis itself coordinates important steps in muscle regeneration.

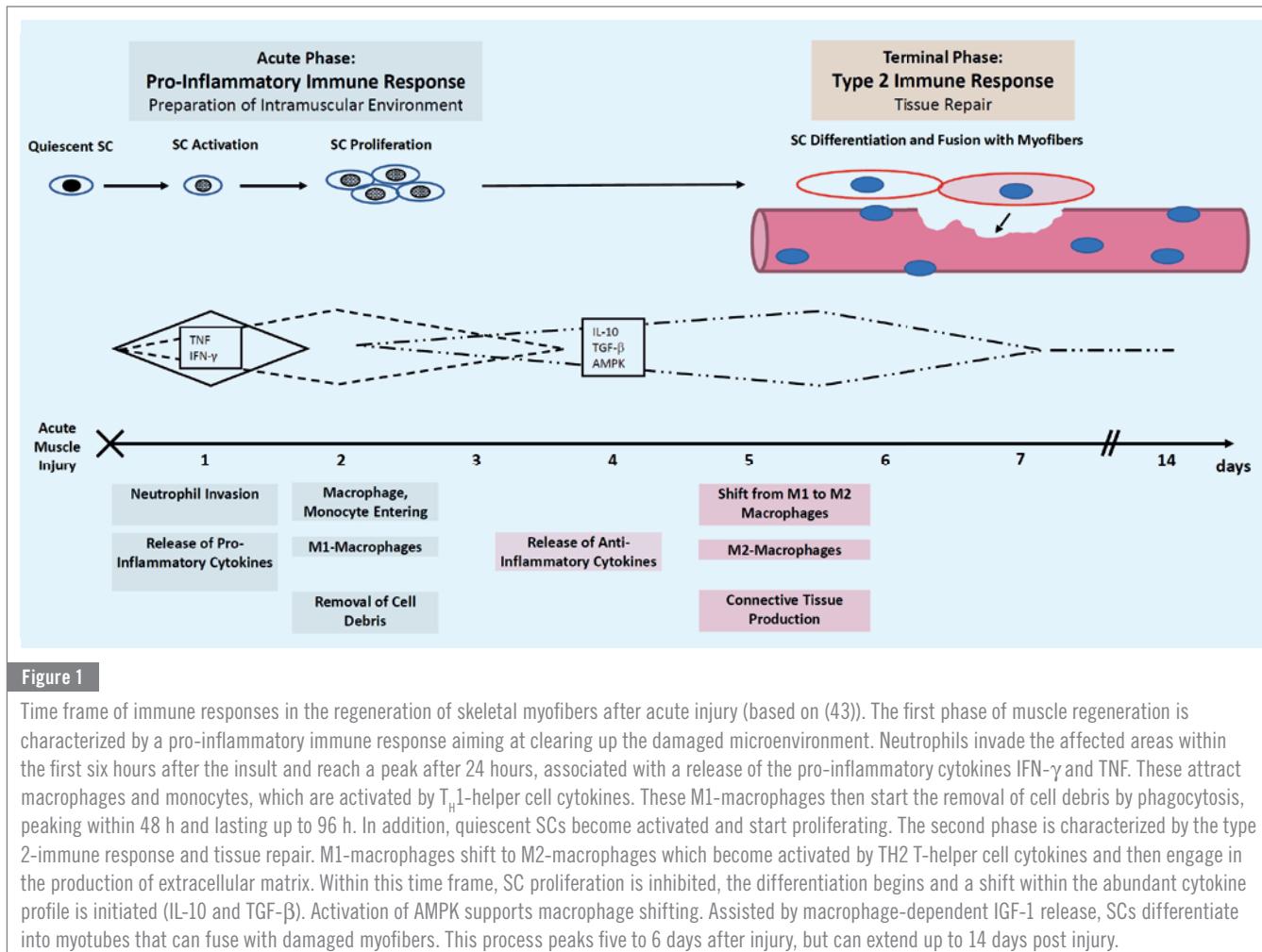
Changes in the expression of cytokines are essential indicators of this mechanism. Increased expression of IL-10 and transforming growth factor- β (TGF- β) accompany the switch from M1 to M2 macrophages several days after injury and indicate the shift from the proliferative state towards increased differentiation and myogenesis (49).

IL-10 inhibits the M1 macrophage phenotype by suppressing the release of pro-inflammatory cytokines and activates macrophages to switch to an M2-dependent phenotype (10). As M2 macrophages induce extracellular matrix synthesis, this mechanism links the immune response upon muscle damage with the preparation for extracellular matrix re-synthesis.

However, IL-10 also regulates macrophage phenotype by controlling mechanisms that are crucially involved in energy metabolism and muscle regeneration. The anti-inflammatory cytokines IL-10 and TGF- β also activate AMPK, which contributes to the shift from M1 to M2 (37). 5-AMP activated kinase (AMPK) is mainly regulated by low energy levels in the cellular environment, but has also regulatory effects on inflammation (54) and on macrophage phenotypes. M1 macrophages are more dependent on glycolytic metabolism whereas M2 macrophages more on oxidative metabolism, which might be the reason why AMPK levels and activity differ between these macrophage types. AMPK co-regulates macrophage-dependent muscle regeneration and can also be attributed to the production of anti-inflammatory cytokines (37). In summary, IL-10 induces M1 macrophage to M2 transitions, which increases AMPK activity. This leads to an AMPK-dependent production of anti-inflammatory cytokines, which supports type 2 immunity.

In addition, at this transition, also the onset of angiogenesis and the rebuilding of capillaries is initiated (20). TGF- β and IGF-1 serve as potent pro-angiogenic molecules (30, 36). During the time frame of M2 macrophage activation, extracellular matrix synthesis, turnover and modulation is an indispensable structural reorganization to generate the necessary space for new blood capillaries (2). M2 but not M1 macrophages promote angiogenesis by modulating fibroblast growth-factor signaling (20).

The expression of cytokines is a major driver in regulating immuno-dependent muscle regeneration. However, also growth factors influence the efficiency of muscle regeneration and macrophage phenotype. Growth factors like IGF-1 can be released by M1 macrophages and constitute potent mitogenic factors for SCs (45).

**Figure 1**

Time frame of immune responses in the regeneration of skeletal myofibers after acute injury (based on (43)). The first phase of muscle regeneration is characterized by a pro-inflammatory immune response aiming at clearing up the damaged microenvironment. Neutrophils invade the affected areas within the first six hours after the insult and reach a peak after 24 hours, associated with a release of the pro-inflammatory cytokines IFN- γ and TNF. These attract macrophages and monocytes, which are activated by T_H1-helper cell cytokines. These M1-macrophages then start the removal of cell debris by phagocytosis, peaking within 48 h and lasting up to 96 h. In addition, quiescent SCs become activated and start proliferating. The second phase is characterized by the type 2-immune response and tissue repair. M1-macrophages shift to M2-macrophages which become activated by TH2 T-helper cell cytokines and then engage in the production of extracellular matrix. Within this time frame, SC proliferation is inhibited, the differentiation begins and a shift within the abundant cytokine profile is initiated (IL-10 and TGF- β). Activation of AMPK supports macrophage shifting. Assisted by macrophage-dependent IGF-1 release, SCs differentiate into myotubes that can fuse with damaged myofibers. This process peaks five to 6 days after injury, but can extend up to 14 days post injury.

Although muscle fibers release IGF-1 themselves, the environment under acute damaging situations might not be suitable for a sufficient paracrine delivery of IGF-1. In this context, the localized release of IGF-1 via macrophages, serves as a more precise mechanism to support muscle regeneration at sites where macrophages have invaded the damaged environment in muscle. The importance of macrophage-derived IGF-1 release for regenerating muscle has been shown by a selective IGF-1 mutation in myeloid cells (45), which resulted in reduced satellite cell numbers. However, IGF-1 can stimulate both, proliferation and differentiation processes both of which are dependent on different signaling pathways (44). While proliferation depends on the IGF-1-receptor/Ras/Raf/MAP Kinase pathway differentiation relies on IGF-1/PI3-kinase/p70s6k signaling (8). Both processes imply unique and time-dependent regulatory patterns for the regenerative process of damaged muscle. In the acute phase, initial IGF-1 release by neutrophils activates Ras/Raf/MAPK kinase signaling to enhance proliferation. At this stage, the autocrine action of IGF-1 induces a shift of macrophages to the regenerative M2 phenotype. This is accompanied by a reduction in pro-inflammatory cytokines. It is believed that with reduced inflammation, fibroblasts may serve as sources for IGF-1 that trigger IGF-1/PI3 kinase/p70s6k signaling to promote differentiation (44). This coordinated and localized release of IGF-1 by macrophages and fibroblasts may have important implications for further therapeutic interventions. As especially the time frame for the proliferative program of SCs after damage is timely limited, therapeutic strategies which efficiently support IGF-1 mediated signaling probably need to be timely well-coordinated (28, 44).

It has been shown that prolonged endurance exercise is associated with an increase in M2 macrophages associated with an increase in IGF-1 mRNA and myofiber hypertrophy (50). Although this study did not prove macrophages to be solely responsible for moderate hypertrophy in response to endurance exercise, it emphasizes a substantial role for immune cells after exercise, even without damaging conditions.

Muscle Regeneration and FAB Cells

FAB cells are fibro-adipogenic progenitor cells that, similar to SCs, reside in a quiescent state in skeletal muscle. Based on the signature of cytokine expression, e.g. during type 2 immune responses, IL-4 and IL-13 expression prevents the differentiation of FAB cells into adipocytes and instead directs them to support myogenesis and muscle regeneration (16). Muscle immune cells interact with FAP cells that induce the production of extracellular matrix components and restore fibrous tissue integrity. These mesenchymal cells express the genes encoding platelet derived growth factor receptor- α and become rapidly activated upon acute injury and exercise (12). Normally, in the resident and quiescent state, they reach peak numbers 72 hours post injury and decline to baseline levels within two weeks, a time course similar to that of macrophages and SCs. The function of these cells is also determined by cytokines expressed by myeloid cells (43). IL-4 produced by eosinophils can shift FAP cells towards the M1 phenotype early after injury (26). They are later eliminated by apoptosis, driven by TNF release, which is expressed by M1 macrophages. A major task of FAP cells in association with myeloid cells is the regulation of the production of

extracellular matrix necessary for muscle regeneration (12). M2 macrophages, which release TGF- β , prevent TNF-induced apoptosis of these cells and support their expansion to the fibrogenic phenotype. The production of extracellular matrix reflects the quality of the regeneration process and the return to basal levels of the integrity of the tissue environment. M2a macrophages support this by producing metabolic components that mediate the generation of proline, essentially required for collagen synthesis (9).

The Importance of Coordinated Steps within the Process of Muscle Regeneration

The entire time frame and the coordinated mechanisms that are described here are adapted to acute damage of skeletal muscle, but not specifically to situations where muscle damage occurs under chronic circumstances (43). Under these conditions, the highly coordinated transitions between phagocytizing and regenerating macrophages as well as the tightly controlled necessity of collagen production stimulated by immune cells, might derange over extended time courses (43). The environment of previously well-arranged tissue constellation might then shift towards increased fibrosis (52) associated with insufficient recovery of tissue integrity. Indeed, at early stages of chronic damage, macrophages can switch to phenotypes which normally do not produce connective tissue. However, in aging muscle, the coordination between muscle and immune cell interaction becomes more ineffective, which contributes to impaired muscle regeneration. Particularly perturbations in signaling between FAP and Treg cells seem to contribute to slower regeneration of damaged muscle. The important rise of Treg cell numbers, due to proliferation and recruitment after acute injury is significantly reduced in ageing muscle. This is likely induced by a reduction of IL-33 production (24) by FAP cells, to date the only known source of IL-33 production.

Pharmaceutical Strategies to Support Regeneration from Muscle Damage

Based on the importance of timely coordinated inflammatory and anti-inflammatory cytokine expression and macrophage function, it can be assumed that NSAIDs may influence the regenerative process of skeletal muscle. NSAIDs exert their function mainly by inhibition of Cyclooxygenase 1 and 2 (COX-1 and COX-2), which are produced by macrophages. Inducible COX-2 mediates the production of prostaglandins, e.g. after injury, and has also been linked to the expression of myogenic genes in SCs (3). COX-2 deficiency has been shown to impair regeneration of muscle (39) emphasizing the fact that acute prostaglandin production after macrophage invasion in response to acute damage, is important to initiate muscle regeneration (17). Therefore, it might be hypothesized that the administration of NSAIDs might impair this pathway and muscle regeneration after damage. Indeed, it has been shown that local injection of NSAIDs blunt the proliferative response of satellite cells in human muscle after eccentric exercise (31). In contrast, extended periods of oral NSAID administration, before and after experimentally induced muscle damage in humans, augmented satellite cell proliferation along with increased extracellular matrix production and faster myofiber repair than without treatment (29).

Thus, it is still discussed whether NSAID application is useful for the treatment of muscle regeneration and there are no consistent recommendations concerning their application in the treatment of muscle injury (19). Based on the important time course of prostaglandine E2 synthesis (PGE2) in the acute phase (48 hours) of muscle injury (17), there are recommendations to apply NSAIDS not sooner than 48 hours after injury (35). Although it has been recently published that NSAIDs are at least useful to reduce strength losses and muscle soreness after exercise (33), there is common sense that much more studies are required to address these questions precisely. However, a recent study, using a muscle damage model in mice, showed that NSAIDS did not significantly affect macrophage phenotype shifting, but importantly impaired muscle metabolism, sarcolemmal repair and induced a pro-apoptotic phenotype (6). ■

Conflict of Interest

The authors have no conflict of interest.

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