Chapter 3
Dependency on Non-myogenic Cells for Regeneration of Skeletal Muscle

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3.1 Introduction

In the search to uncover the mechanisms of tissue regeneration and how they can be leveraged for therapeutic approaches, skeletal muscle has become an attractive model. Studies in the genetically tractable mouse have provided insight into the myogenic progenitor cells and signaling networks essential for efficient muscle repair in response to acute and chronic damage. More recently, it has become clear that crosstalk between muscle, the innate immune response and interstitial fibroblastic cells is essential for muscle regeneration. An imbalance in signaling, as observed with chronic inflammation of Duchenne's muscular dystrophy patients, can lead to a progressive increase in fibrosis, fat deposition and muscle necrosis. In contrast, de novo muscle regeneration in response to amputation or severe trauma is largely limited to amphibians, reptiles, and fish among the vertebrates. The additional layers of regulation are necessary to recruit progenitor cells to the site of the amputation as well as impose the positional identity required to accurately regenerate individual muscle groups. Similarly, myeloid and fibroblastic cells have also been shown to participate in these processes. In this chapter, we will review the recent advances in our understanding of the role of non-myogenic cells in muscle regeneration.

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J. Wilson-Rawls, K. Kusumi (eds.), *Innovations in Molecular Mechanisms and Tissue Engineering*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-3-319-44996-8_3

3.2 Satellite Cells of the Myogenic Lineage

Skeletal muscle regeneration is dependent on satellite cells that are functionally defined by their ability to both self-renew and differentiate into myoblasts that are able to fuse to form myofibers. These cells are maintained in a quiescent (G_0 phase) state until environmental cues associated with muscle injury stimulate re-entry into the cell cycle. During effective muscle repair, activated satellite cells migrate to the site of injury, proliferate, and differentiate to generate new muscle fibers.

Satellite cells are characterized by their location beneath the basal lamina of muscle fibers and constitutively express the transcription factors *Pax7* and *Myf5* [1, 2]. Ablation of *Pax7* results in decreased satellite cell proliferation and self-renewal, significantly impacting muscle growth and repair [2]. Quiescent satellite cells (QSCs) have been found to express 500 genes not present in activated satellite cells that participate in cell–cell adhesion, negative regulation of the cell cycle, transcriptional control, and lipid and extracellular matrix transporter activity [3]. Gene loci in QSCs that are only expressed at very low levels until induction via the onset of satellite cell activation are marked by histone H3 Lys4, a marker of active chromatin, indicating that these regions are open, awaiting the signals necessary to prompt activation and begin repair, and not in a dormant state [4, 5]. The ability of QSCs to immediately respond to injury stimuli allows for effective muscle repair.

Upon muscle injury, the myofiber sarcolemma and basal lamina are dismantled, resulting in a disconnection between satellite cells and the collagen-laminin network on which they are anchored. This disruption of the myofiber allows for the release and entry of factors critical for satellite cell activation. One of the first factors implicated in activation, hepatocyte growth factor (HGF), is released from the basal lamina, it then proceeds to bind to the Met receptor on the surface of satellite cells, causing their activation and aiding in their migration to the injury site [6]. Dying fibers within the niche generate nitric oxide (NO), further stimulating HGF release from the basal lamina. Also implicated in the activation and proliferation of satellite cells is the Notch signaling pathway; blockage of Notch leads to inhibition of satellite cell proliferation, whereas up-regulation of Notch leads to the promotion of muscle regeneration [7, 8]. In the muscle niche itself, several factors are secreted that aid in multiple aspects of muscle repair. Fibroblast growth factor (FGF) secretion into the ECM activates the MAPK cascade, resulting in the activation and regulation of satellite cell quiescence [9]. Phosphorylated p38 and MyoD are among the earliest markers of activation, with p38α/β MAPK inducing MyoD protein expression. In support of satellite cell proliferation, Notch3 mRNA and protein levels decline upon activation [10]. Additionally, production of the MYF5 protein begins due to a decrease in miR-31 levels, giving activated satellite cells a $Pax7^{+}$, $Myf5^{+}$ phenotype.

Recently, an additional phase of satellite cell quiescence, termed the G_{alert} phase, has been identified in response to injury. Experiments performed by Rodgers et al. [11], demonstrated that satellite cells residing in muscle in the leg contralateral to the limb with the induced injury were distinct from both quiescent and activated

satellite cells. In culture, QSCs in the G_{alert} phase were found to enter the cell cycle earlier than non-injury-induced QSCs. Additionally G_{alert} phase QSCs demonstrated an increase in cell size as compared to QSCs, and a high transcriptional correlation between G_{alert} phase QSCs and activated satellite cells was identified. Both mTORC1 activity and HGF signaling were required for QSCs to switch from G_0 to the G_{alert} phase in response to injury. These findings suggest that G_{alert} phase QSCs retain properties of both QSCs and activated satellite cells in a phase that is "primed" for injury response. In fact, QSCs of the G_{alert} phase demonstrated heightened differentiation in culture and enhanced regeneration following an induced injury in vivo [11].

3.2.1 Proliferation of Satellite Cell and Myoblasts

Satellite cell activation is followed by the rapid expansion of *Pax7*$^+$, *Myf5*$^+$ cells that will form the myoblast population, eventually participating in muscle repair, and self-renewal of a smaller population of *Pax7*$^+$, *Myf5*$^-$ satellite cells that will become quiescent in anticipation of later injury events (Fig. 3.1). The majority of *Pax7*$^+$, *Myf5*$^+$ satellite cells undergo symmetric division, producing two *Pax7*$^+$, *Myf5*$^+$ progenitor cells. WNT7a, acting through its receptors FZD7 and VANGL2, induces symmetric cell division through the planar cell polarity pathway [12]. In addition to HGF, insulin-like growth factor-1 (IGF-1), epidermal growth factor (EGF), transforming growth factors α/β (TGFα and TGFβ), and platelet-derived growth factor (PDGF) also contribute to the proliferation and differentiation of myoblasts [13]. Due to damage of the sarcolemma and basal lamina, myofibers receive an inflow of calcium from the (ECM) matrix, which aids in proteolysis of the myofiber [14]. *Pax7*$^+$, *Myf5*$^+$ cells, stimulated through activated leukocyte secretion of IGF-1 and delivered through capillaries into the niche, will continue to proliferate through the down-regulation of $P27^{kip1}$ and through inactivation of the transcription factor FOXO1 [15]. Negative mitogenic modulation of satellite cells exists through the transforming growth factor β (TGFβ) superfamily, most notably myostatin, which inhibit differentiation of satellite cells through down-regulation of MyoD expression and inhibits activation through the up-regulation of P21 and decreased levels of CDK2 [16, 17]. Tumor necrosis factor α (TNFα) also negatively mediates differentiation through the utilization of the TGFβ activated kinase (TAK1)/p38/NF-kB pathway, resulting in increased levels of Activin A expression to support proliferation [18].

Approximately 10 % of the satellite cell population maintains a *Pax7*$^+$, *Myf5*$^-$ profile and will undergo asymmetrical division to give rise to one *Pax7*$^+$, *Myf5*$^-$ and one *Pax7*$^+$, *Myf5*$^+$ cell (Fig. 3.1). Several signaling pathways present in the microenvironment of the satellite cell niche are responsible for controlling asymmetric satellite cell polarity and fate. Components of the Notch pathway, including a Notch3 effector protein, Notch ligand Delta1 (Dll1), and Notch agonist Numb have all been found to asymmetrically distribute between daughter cells, with

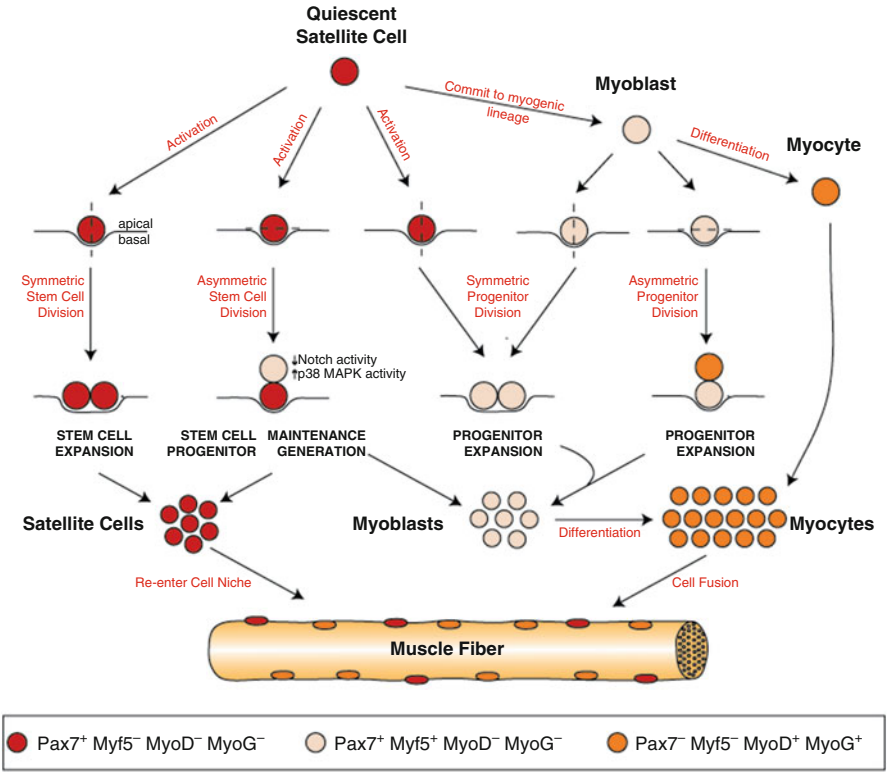

Fig. 3.1 Mechanisms of satellite cell division for muscle maintenance and repair. Following entry to the cell cycle, quiescent satellite cells symmetrically or asymmetrically divide along the apical-basal axis. Symmetric and asymmetric divisions lead to the generation of additional muscle stem cells and progenitor cells. Additionally, satellite cells can directly commit to the myogenic lineage and expand the progenitor cell population or differentiate into myocytes. Resulting muscle stem cells return to the niche to replenish the pool of quiescent satellite cells. Resulting myocytes fuse to form myotubes, leading to the formation of new muscle fibers

DLL1 and NUMB found selectively in the daughter cell committed to becoming a myoblast [8, 19]. Ablation of *Numb* in the muscle lineage profoundly decreased satellite cell proliferation, negatively affecting the ability of muscle to repair following an induced injury [20]. Additionally, factors involved in cell polarity determination, namely parts of the Par complex and Scribbled planar cell polarity protein (Scrib), have been implicated in asymmetric division. Orientation to the myofiber plays an important role in the ability of the satellite cells to asymmetrically divide. This relation to the myofiber, conferred by an apical-basal polarity, is dependent on the interaction of cell membrane receptors basal integrin α7β1 and apical M-cadherin, resulting in the production of one basal *Pax7*$^{+}$ *Myf5*$^{-}$ cell and one apical *Pax7*$^{+}$ *Myf5*$^{+}$ daughter cell [8]. It has also been proposed that the position of the mitotic spindle in relation to the myofiber axis plays a role in asymmetric division cell fate [21].

3.2.2 Heterogeneity of the Satellite Cell Population

Studies in culture first revealed heterogeneity in the satellite cell population with a "responsive population" that readily proliferates in response to damage and participates in repair, and a "reserve population" that divides at a slow rate and is refractory to differentiation into mature myotubes. This heterogeneity has been reported in muscle tissue at a ratio of 5:1 (responsive: reserve), confirming their relevance to normal muscle biology. The slow dividing cells contribute solely to skeletal muscle when transplanted back into mouse EDL muscle, confirming their commitment to the myogenic lineage. Genome-wide gene expression studies revealed differential expression between the two populations with reserve cells expressing higher levels of inhibitor of differentiation (Id) and other genes that confer "stemness". This predicts that the slow dividing cells that are refractory to repair signals, are essential to muscle homeostasis for long-term maintenance of the satellite cells population.

3.3 Satellite Cell Regulation Through the Stem-Cell Niche

The activation, migration, and proliferation of satellite cells are supported by the inflammatory microenvironment created by components of the niche and immune cells. In addition to ECM, the niche includes fibro-adipogenic (FAP) cells, vasculature, and both residential and infiltrating immune cells that are capable of direct communication with satellite cells. Oxygen free radicals released by neutrophils further break down the sarcolemma, while matrix metalloproteinases released by both damaged myofibers (MMP2) and immune cells (MMP9), aid in the degradation of ECM proteins [22]. ECM digestion through MMPs plays a vital role in satellite cell migration to the site of injury, especially in fibrotic tissue.

FAPs are bipotent fiber-associated cells that also proliferate in response to muscle fiber injury [23]. FAPs double in number in less than 48 h and up-regulate the expression of Interleukin 6 (IL-6) roughly tenfold. IL-6, along with Wnt and IGFs, has been implicated as a pro-differentiation signal that is essential for the differentiation and maturation of myoblasts during muscle repair [23–25]. During myolysis, FAPs have been found to assist in the clearing of cellular debris through phagocytosis of necrotic thymocytes, and when compared to macrophages, FAPs have been found to be fourfold more efficient in debris clearance [26].

Microvasculature and accompanying pericytes help to sustain the cells of the microenvironment, as well as provide the necessary access to circulation for immune cell infiltration in response to damage and delivery of key factors that assist with niche maintenance and satellite cell regulation [4, 17, 27]. PDGF and vascular-endothelial growth factor (VEGF) are released from ruptured blood vessels in response to injury and play an important role in reciprocal communication with satellite cells to promote their proliferation, as well as angiogenesis [28].

Satellite cells are commonly found surrounding the vasculature within a 5 μm radius, with up to 82 % in murine models and 68 % in human residing near capillaries [29]. Pericytes in the muscle serve a jack of all trades role; they help to replace and regenerate the vasculature that can be lost or damaged due to muscle injury, also have been found to replace muscle, and become myogenic in vitro [30]. Pericytes have also been shown to give rise to most of the collagen forming cells during muscle injury, and, in the presence of neurons, have been shown to produce collagens I and III [31].

The ECM contributes to the regulation of satellite cells in the niche. Proteoglycans and glycoproteins play a role in niche homeostasis and in the repair process. Collagen VI ablation in mice leads to a muscle wasting disease not dissimilar to the common dystrophic models [32]. ECM proteins bind to the transmembrane protein dystrophin, forming an anchor that connects the satellite cells to the basal lamina and maintains their anatomical location [33]. ECM proteins can also act as mitogens for satellite cells. Resting, non-damaged satellite cells are located in fibronectin rich regions of the myofiber niche, Syndecan4 (SYN4) and Frizzled7 (FZD7) on the satellite cells act as co-receptors to bind fibronectin [34]. In the presence of WNT7a, this complex will induce symmetrical division. Upon muscle damage, fibronectin is transiently expressed to help maintain the satellite cell pool through the Wnt signaling pathway [34, 35].

The elasticity of the myofiber also plays a role in regulation; normal muscle fibers have a Young's modulus of approximately 12 kPa, while those in aged or dystrophic muscle are much stiffer [36, 37]. This leads to a decrease in quiescent satellite cells because the increased stiffness induces them to enter the cell cycle. Recent work using collagen based scaffolds with elasticity from 2 to 25 kPa as determined by atomic force microscopy (AFM), has shown that on substrates that measure 2 kPa most of the satellite cells maintain their quiescent states and do not enter the cell cycle. Whereas at 25 kPa only about 45 % remain quiescent in vitro [38]. These findings could explain why in aged or dystrophic muscle there is a decreased satellite cell presence, as these two niche environments have an increased stiffness [38, 39].

3.4 Innate Immune Response During Skeletal Muscle Repair

Regeneration of skeletal muscle cannot be accomplished solely by satellite cells. Several types of immune cells, both resident and infiltrating, play an indispensable role in effective tissue regeneration. In healthy homeostatic muscle, immune cells are kept at a minimum, however, disruption of the basal lamina and sarcolemma of myofibers initiates several waves of immune cell infiltration that play discrete roles in the removal of necrotic fibers, activation of satellite cells, and ultimately the efficient differentiation into mature muscle fibers. The majority of the immune cells involved in muscle repair are those of the innate leukocyte lineage—macrophages, neutrophils, dendritic cells, mast cells, eosinophils, basophils, and natural killer

cells. Central to the innate immune response is the production and responsiveness to cytokines, chemokines, and growth factors. These signaling molecules mediate crosstalk with satellite cells and FAP cells during the repair process.

Immediately upon myofiber damage, resident mast cells within the muscle degranulate, releasing TNFα, while resident macrophages release C-C motif chemokine ligand 2 (CCL2) and C-X-C motif chemokine ligand 3 (CXCL3), recruiting transient polymorphonuclear neutrophils from the circulation to the site of injury [40]. Satellite cells also contribute to chemoattraction to the site of damage through the release of the pro-inflammatory cytokines IL-1, IL-6, and TNFα [41]. Neutrophils rapidly invade the injured tissue in significant numbers and persist in the tissue for approximately 24 h, where they promote sarcolemma damage through the release of oxygen-free radicals [42]. Through the secretion of IL-1 and IL-8, neutrophils promote the recruitment of circulating $CX3CR1^{low}$, $Ly6C^{+}$, $CCR2^{+}$ phenotype monocytes to the site of injury [43] and binding of CCL2 and CCL7, by the C-C motif chemokine receptor, CCR2 [44]. Disruption of either receptor or ligands leads to severe deficits in monocyte recruitment and efficient muscle repair [45–47]. The infiltrating monocytes differentiate into macrophage subtypes, both pro- and anti-inflammatory, in a process that is highly dependent on the tissue microenvironment.

At approximately 24-h post muscle injury, monocytes/macrophages begin to express high levels of IL-6, supporting macrophage infiltration and myoblast proliferation through the STAT3 pathway. Effective muscle repair requires sufficient generation of myoblasts for regeneration of the damaged tissue. Knockout of IL-6, or knockdown of STAT3, resulted in decreased MyoD, Myogenin, and macrophage infiltration, ultimately resulting in diminished muscle repair [48].

Initially, the pro-inflammatory phenotype is maintained as neutrophils secrete Th1 inflammatory cytokines, interferon-gamma (IFNγ) and TNFα, to induce monocytes to polarize into M1 macrophages ($CX3CR1^{low}$, $Ly6C^{+}$, $CCR2^{+}$). In addition to IFNγ and TNFα, pathogens and granulocyte macrophage colony-stimulating factor (GM-CSF) are capable of stimulating M1 macrophage polarization [49] (Fig. 3.2). M1 macrophages phagocytose cellular debris and secrete factors, such as IL-1b and IL-12, to recruit additional inflammatory cells for debris clearance and pathogen removal. Nitric oxide (NO), produced by M1 cells acts to lyse cells for removal, however, if dysregulated, it can lead to increased tissue damage [50]. During the pro-inflammatory phase, which occurs approximately 24–96 h post injury, the NF-kB pathway in both macrophages and myoblasts is activated in response to TNFα. In macrophages, this enhances the inflammatory response by stimulating the release additional pro-inflammatory cytokines. In muscle, CyclinD1 expression is induced, while MyoD expression is suppressed, in response to activation of the NF-kB pathway, supporting myoblast proliferation and preventing differentiation [51, 52].

Phagocytosis by M1 macrophages and exposure to CSF-1 induce macrophage polarization to skew from a pro-inflammatory phenotype towards an anti-inflammatory phenotype, resolving the inflammation and beginning the muscle repair process [53]. Infiltrating monocytes now become $CX3CR1^{hi}$, $Ly6C^{-}$, $CCR2^{-}$

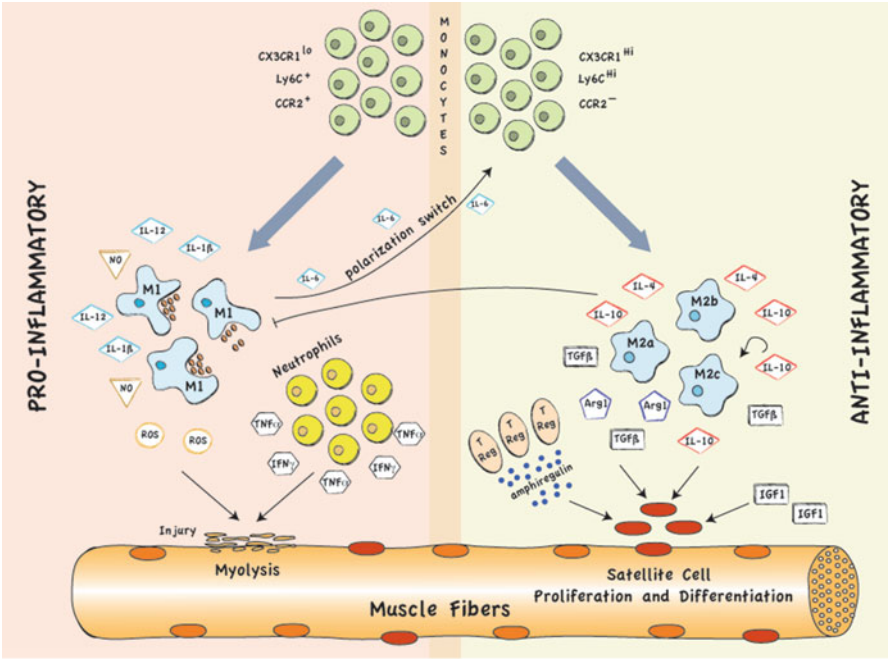

Fig. 3.2 Immune cell contribution and modulation in damaged muscle tissue. In response to myofiber injury, neutrophils from circulation invade the site of damage where they aid in further tissue break down and recruit CX3CR1^{Lo}, Ly6C^{+}, CCR2^{+} monocytes, differentiating into M1 macrophages, for continued debris clearance and pro-inflammatory cytokine secretion. M1 phagocytosis induces macrophage polarization towards an anti-inflammatory phenotype to support muscle repair. CX3CR1^{Hi}, Ly6C^{Lo}, CCR2^{-} monocytes differentiate into M2a, M2b, and M2c macrophages, functioning to suppress inflammation and promote satellite cell proliferation and differentiation. T regulatory cells assist M2 macrophages in resolving inflammation and fostering muscle repair

and differentiate into three subtypes of M2 macrophages. Several molecules have been identified as regulators of the switch from early pro-inflammatory to late anti-inflammatory macrophage phenotypes. cAMP response element-binding protein (CREB), a multifunctional transcription factor, is critical for the up-regulation of genes associated with M2 macrophages (IL-10, IL-13R, Arg-1) and repression of M1 macrophage activation [54]. Mitogen-activated protein kinase (MAPK) phosphatase-1, through inhibition of p38 MAPK activation, functions to control macrophage subtype shifting. MAPK also helps to resolve inflammation to allow for proper muscle repair [55]. Recently, AMP-activated protein kinase (AMPK), widely known as a regulator of metabolic homeostasis, has also been identified as a regulator of macrophage polarization skewing. Mounier et al. [56], demonstrated loss of M2 macrophage functionality and a loss of M2 markers expressed in AMPK$\alpha1^{-/-}$ macrophages. Further, AMPK$\alpha1^{-/-}$ mice showed deficient muscle repair resulting from a failure of M1 macrophage phagocytosis-induced polarization to an M2 phenotype [56].

M2a macrophages arise from the release of IL-4 or IL-13 and signal via IL-4 receptor alpha [57]. Release of these Th2 inflammatory cytokines causes increased expression of CD206 and CD36 by macrophages. In vitro, it has been shown that M2a macrophages, producing arginase, decrease M1 macrophage lysis activity through competition for arginine, the shared enzymatic substrate of arginase and iNOS [58]. M2a macrophages secrete IL-10 and TGF-β, thereby inducing the anti-inflammatory M2c macrophage subtype, which aids in IL-10 and TGF-β release (Fig. 3.2). Secretion of these cytokines suppresses inflammation and promotes satellite cell proliferation, allowing for remodeling of the extracellular matrix, angiogenesis, and muscle fiber development to begin [58]. Glucocorticoids and IFNβ can also stimulate the induction of the M2c subtype [59]. The release of IL-4 by M2b regulatory macrophages, Th2 cells, eosinophils, and basophils further promotes the wound healing phase by decreasing phagocytosis and stimulating macrophage fusion [49]. In addition to IL-4, the release of IGF-1 also contributes to continued satellite cell growth and myofiber fusion [60]. In recent experiments by Tonkin et al. [61], macrophages were identified as a major contributing source of IGF-1 at the site of muscle damage. Indeed, when muscle injury is induced in mice devoid of IGF-1 in myeloid cells, a loss of regenerative capacity is demonstrated. During the late stages of healthy muscle repair, $Ly6C^+$ monocytes/macrophages and $CD206^+$ macrophages were found to express high levels of IGF-1. However, when IGF-1 is knocked out from myeloid cells, the population of $Ly6C^+$ monocytes/macrophages is heightened while the population of $CD206^+$ macrophages is diminished [61].

Aiding in the establishment of the anti-inflammatory environment at the site of muscle damage, a population of $CD4^+$ regulatory T cells (T_{reg}) arises concurrently with M2 macrophages, though to a much lesser extent (Fig. 3.2). FoxP3, a forkhead transcription factor, regulates T_{reg} cell lineage specification, however, it remains unclear whether the population of T_{reg} cells at the site of muscle injury derives from resident T_{reg} cells in the muscle or is recruited in response to damage. T_{reg} cells have been shown to influence myeloid and T cell infiltration, as well as satellite cell colony-forming capacity. Additionally, T_{reg} cells were found express IL-10 and amphiregulin, which accumulate during the final stages of muscle repair and play important roles in negative regulation of inflammation and satellite cell activation and proliferation, respectively [62]. Due to the capability of T_{reg} cells to modulate the inflammatory response and satellite cell activity, research in using T_{reg} cells to improve muscle repair is of current interest. Villalta et al. demonstrated increased levels of T_{reg} cells in both human Duchenne's muscular dystrophy (DMD) and in the corresponding *mdx* mouse. When T_{reg} cells are depleted from dystrophic muscle, a heightened Th1-cell-mediated response occurs causing increased myofiber damage [63].

In recent years, the multi-faceted role of macrophages in wound repair has begun to lend itself to potential use in therapy for muscle injury. M1-polarized macrophages delivered to the site of muscle damage resulted in enhanced recovery of functionality with reduced myofiber damage and collagen accumulation [64]. When M2a or M2c macrophages are injected, an increase in tube-like structures is observed, indicating improved angiogenesis [65]. To further aid in the repair of

muscle injury, especially in cases of volumetric muscle loss, tissue scaffolds with inert or biodegradable properties have been the predominating focus. Contrary to avoiding an immune response, recent work has sought to take advantage of immune cells in the delivery of tissue scaffolds—now termed "smart scaffolds". Macrophages and other inflammatory cells, such as cytokines capable of modulating macrophage polarization, can be loaded into tissue scaffolds prior to transplantation, allowing for a therapeutic approach that is personalized and works in conjunction with the patient's own immune response to enhance the repair process. Through an injectable multidomain peptide scaffold engineered by Kumar et al. the potential to recruit specific inflammatory cells and deliver cytokines to the site of injection was shown. MCP-1 and IL-4 loaded hydrogel scaffolds were capable of boosting macrophage recruitment and stimulating polarization towards a pro-healing M2 phenotype in a time-controlled manner, without inducing a local inflammatory response [66].

3.5 De Novo Regeneration of Skeletal Muscle

As described above, mammalian models have been powerful tools in parsing the signaling pathways regulating the regeneration of skeletal muscle in response to acutely damaged muscle. However, de novo muscle regeneration in response to amputation is largely limited to amphibians, reptiles and fish among the vertebrates. This process can be distinguished by the additional layers of regulation necessary to recruit progenitor cells to the site of the amputation and a complex set of temporal and spatial signals necessary to impose the positional identity required to accurately recapitulate individual muscle groups and coordinate the regeneration of distinct cell lineages that give rise to the skeletal elements, connective tissue, nerves, vasculature, and skin [67]. As with tissue repair, the study of skeletal muscle regeneration has been central to our understanding of complex tissue regeneration. Non-myogenic cell types have been implicated in this process. In this section, we will compare the regulation of muscle repair to regeneration through the lens of the microenvironment created by the immune cells and myofibroblasts.

3.5.1 Amphibians as a Model for the Study of Skeletal Muscle Regeneration

Members of the Anura (frogs and toads) and Caudata (salamanders and newts) orders are the most commonly studied amphibians for muscle regeneration. Anurans possess distinct developmental windows preceding metamorphosis where complete regeneration of organs can occur, while the urodeles (Caudata) are able to regenerate a wide variety of organs throughout adulthood. Perhaps the best studied regenerative tissue system has been limb and tail amputations that follow a

conserved set of temporal events that include (1) a modified wound healing process, (2) progenitor cell recruitment and (3) activation and tissue rebuilding (reviewed in [67–69]). Conserved regulatory pathways shared between amphibian models has provided insight into how regeneration has been maintained in these animals and largely lost in mammals.

3.5.2 Wound Healing and ECM Remodeling During Regeneration

Wound healing associated with regeneration shares many common features with scar-free wound healing associated with skin repair. Within hours of amputation, epithelial cells and dermal fibroblasts migrate to the site of injury and cover the fibrin blood clot. The regenerative epithelial cells thicken to form an apical ectodermal cap (AEC) reminiscent of the apical ectodermal ridge (AER) that appears during limb development. The AEC promotes the remodeling of the basement membrane ECM through recruitment of leukocytes and the release growth factors that are capable of inducing the subjacent mesenchymal cells to form a blastema of undifferentiated proliferating progenitor cells with the ability to rise to the distinct cell types of the limb [70–72]. In the case of skeletal muscle, progenitor cells can be derived from myoblasts ($Pax7^{-}$, $MyoG^{+}$) that dedifferentiate muscle fibers and aid in the recruitment of satellite cells ($Pax7^{+}$, $MyoG^{-}$) [73, 74].

The ECM at the site of the wound is recognized as an important regulator of wound healing and the progression towards regeneration. ECM is a complex network of proteins composed primarily of collagens, laminins and fibronectins that interact to create scaffolding as well as serve as adhesion sites for cells through integrin binding. Small leucine-rich proteoglycans within the ECM bind growth factors and cytokines that create microenvironment niches for cell signaling [75]. Within hours of amputation, migrating epithelial cells express matrix metalloproteinases (MMP) that promote ECM breakdown through the digestion of collagen. This facilitates cell invasion, debris clearance and release of the growth factors and cytokines that promote cell migration [72, 76]. A second wave of MMP expression after 3 days is believed to participate in ECM remodeling and promoting muscle dedifferentiation [77]. Treating newt wounds with MMP inhibitors resulted in shortened stumps with distal scars, indicating the importance of the ECM remodeling during regeneration [78]. Macrophages represent important regulators of ECM breakdown and remodeling at the wound site. Inflammatory cytokines produced by macrophages regulate ECM production from fibroblasts and myofibroblasts and ensure a pro-regenerative microenvironment at the site of the wound instead of an acellular fibrotic scar [79, 80]. Depletion of macrophages in salamanders inhibits limb regeneration and promotes the formation of a distal scar and an overrepresentation of myofibroblasts [81]. This underscores the important relationship between the organism's ability to remodel ECM and the formation of fibrotic scars that prevent regeneration. In support of this, salamanders maintain the expression of other

developmentally regulated collagens III and XII, tenascin, and hyaluronic acid later into adulthood than mice and delay the onset of collagen I that gives rise to acellular scars through cross-linking with heparin sulfate proteoglycans [81, 82].

3.5.3 Myogenic Progenitor Cells During Regeneration

In classic experiments initially performed in salamanders, myogenic progenitor cells contributing to the blastema were found to be derived through the dedifferentiation of injured muscle [83–85]. Dedifferentiation is characterized by a loss of differentiated muscle-specific markers, fragmentation of multinucleated myotubes into mononucleated cells and re-entry into the cell cycle [86]. The resultant mononucleated $Pax7^{-}$ $MyoG^{+}$ cells are capable of redifferentiation into muscle [87]. Cre-loxP-based genetic fate mapping experiments have demonstrated that cells generated through dedifferentiation remain restricted to the myogenic lineage and are unable to contribute to other tissues of the limb or tail [68, 88].

Several transcription factors and cell cycle regulators have been shown to regulate muscle dedifferentiation [74, 89–91]. Perhaps the best studied are members of the MSX family of the homeodomain-containing transcription factors (MSX1 and MSX2) that have been implicated in maintaining cells in proliferative, progenitor state during limb development across vertebrates. Over expression of either MSX1 or MSX2 is sufficient to drive myotube dedifferentiation in culture and the formation of differentiation-competent myoblasts [90]. More recently, it was found that the LIM homeobox transcription factor, *Lhx2*, which can suppress muscle-specific transcription and differentiation in C2C12 cells, is a direct regulator of Msx1 and Msx2 transcription [92]. Further, ectopic expression of MSX1 or MSX2 can induce dedifferentiation of mammalian myotubes suggesting the elements of the dedifferentiation regulatory network of the amphibians have been retained in mammals [93–95].

Inactivation of the tumor suppressor Retinoblastoma (Rb) through phosphorylation has also been implicated in muscle regeneration in the newt limb, consistent with the requirement for reinitiating the cell cycle during generating progenitor cells [74]. Inactivation of Rb is sufficient to promote DNA synthesis in differentiated mouse muscle in culture, however, the cells will not progress to proliferating myoblasts with the capacity for redifferentiation [96, 97]. Complete recapitulation of the dedifferentiation pathway requires an additional insult to the p53 signaling pathway through inactivation of the Alternate Reading Frame (ARF) of the *Ink4a* locus [91]. Interestingly, the earliest identified *ARF* ancestor is in chickens, with no candidates in databases for non-amniote organisms [98–100]. This raises the possibility that loss of regenerative capacity in mammals is related to acquisition of additional levels of cell cycle regulation. There is evidence that environmental cues participate in the regulation of muscle fiber dedifferentiation. The ECM in the tissue proximal to the site of amputation undergoes a shift from a collagen and laminin-based stiff ECM to a softer transitional ECM rich in hyaluronic acid, tenascin-C and fibronectin. Under cell culture conditions, this ECM differentially directs DNA synthesis, migration, myotube fragmentation and myoblast fusion [101, 102].

In addition to the generation of *Pax7*$^{-}$, *Myog*$^{+}$ myoblasts through dedifferentiation, there is evidence that recruitment of *Pax7*$^{+}$, *Myog*$^{-}$ satellite cells from muscle proximal to the site of amputation participates in muscle regeneration in salamanders [73]. Further, cultured satellite cells are able to contribute to muscle regeneration upon transplantation [68, 103]. This indicates that the system for recruiting myogenic progenitor cells in mammals can participate in regeneration in amphibians as well. Cre-loxP-based genetic fate mapping approaches have been used to track cells in the blastema that are *Pax7*$^{-}$, *Myog*$^{+}$ and *Pax7*$^{+}$, *Myog*$^{-}$ [68, 88]. Surprisingly, there was a preference for the recruitment of a premyogenic cell source between urodeles, with the *Notophthalmus viridescens* (newt) depending on dedifferentiation of muscle while the *Ambystoma mexicanum* (axolotl) leverages satellite cells [88]. The newt employs a dedifferentiation strategy for the regeneration of other tissues, including the lens of the eye, while the axolotl has limited regenerative capacity for the lens [104, 105]. This reveals a divergence in strategies for generating progenitor cells for tissue of two urodeles separated by approximately 100 million years. This raises interesting questions about the evolutionary pressures that would maintain two discrete mechanisms. The selection process has been strong enough that mammalian muscle is able to functionally recapitulate dedifferentiation with relatively small changes in gene expression of extracellular matrix.

3.5.4 Role of Pro- and Anti-Inflammatory Immune Response in Regeneration

The duality of the innate immune response with the pro-inflammatory arm directed by Th1 cytokines and the anti-inflammatory arm directed by Th2 cytokines is conserved in urodeles. However, analysis of the cytokines post limb amputation reveals two overlapping spikes in Th1 and Th2 cytokines as well as CCL and CXCL chemokines at days 2 and 7, which predicts that anti-inflammatory M2 macrophages are recruited concurrently to the site of injury with pro-inflammatory M1 macrophages [81]. This is in contrast to mammalian muscle repair, where a distinct early wave of pro-inflammatory M1 macrophages is followed by anti-inflammatory M2 macrophages. The presence of M2 macrophages and Th2 cytokines did not inhibit the phagocytic activity of M1 macrophages in the first 24 h post-amputation in the salamander, suggesting a different functional relationship between the two cell types during regeneration. Interestingly, M1 macrophage activity requires expression of anti-inflammatory cytokines as well as several signalling pathways critical for regeneration, including metalloproteinases MMP9 and MMP3, dedifferentiation regulator *Msx2*, blastemal markers *Prrx1* and *Sp9*, the production of Th2 cytokines, and TGFβ signaling [81]. Thus, despite the temporal overlap, modulation of the pro-inflammatory immune response is essential for promoting regeneration.

Studies in Anurans, where regenerative capacity is limited to a pre-metamorphosis time period provides an opportunity to compare cellular processes associated with repair in permissive and non-permissive stages to examine mechanisms by which

the immune system regulates regeneration (reviewed in [106]). *Xenopus*, the most common anuran model, will undergo complete limb or tail regeneration between pre-metamorphosis stages 50–53. After metamorphosis has started (stages 57–60), regeneration is only partially complete as exemplified by a cartilaginous spike replacing an amputated limb. The shift from tadpole to adult is associated with immunological shifts from a relatively simple "ancestral" system to one that is more complex and resembles that of the mammals [107, 108]. Consistent with this, differential gene expression studies between regeneration competent and incompetent stages confirms differences in the immune signaling and resolution of inflammation [109–111]. While pro-inflammatory signals spike early after limb amputation in stage 53 of *Xenopus*, they persist at the regeneration non-competent stage 57 [110]. This would indicate that unresolved inflammation in response to injury contributes to the loss of the regenerative capacity in adult frogs. In support of this, immune cell depletion can extend the period of regeneration competence in *Xenopus* [112].

Studies in anurans and urodeles have provided seemingly conflicting models of the role of the inflammatory response to regeneration, with disruption of inflammatory macrophages inhibiting salamander and newt regeneration while extending the regenerative refractory period in frogs [81, 112]. This can best be reconciled through the lens of comparative strength of the immune system. Salamanders are considered to have a strong innate immune system, but because of the lack of key adaptive immune responses, it is considered relatively weak compared to the frog and mouse [113]. In the case of the frog, the strength of the immune system increases with age, leading to the hypothesis that the regenerative capacity of the organism is inversely proportional to the strength of the immune response to injury. This is likely an oversimplified axiom as phagocytotic macrophages are essential for salamander limb and tail regeneration. There has been considerable effort to understand the immune response to pathogens and this can provide insight into differences in humoral and cytotoxic immune response between amphibians [72, 114, 115]. Understanding how the broader immune system plays a role in tissue regeneration should help resolve this confusion.

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