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Myeloid cell diversification during regenerative inflammation: Lessons from skeletal muscle

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Abstract

Understanding the mechanisms of tissue and organ regeneration in adult animals and humans is of great interest from a basic biology as well as a medical, therapeutical point of view. It is increasingly clear that the relatively limited ability to regenerate tissues and organs in mammals as oppose to lower vertebrates is the consequence of evolutionary trade-offs and changes during development and aging. Thus, the coordinated interaction of the immune system, particularly the innate part of it, and the injured, degenerated parenchymal tissues such as skeletal muscle, liver, lung, or kidney shape physiological and also pathological processes. In this review, we provide an overview of how morphologically and functionally complete (*ad integrum*) regeneration is achieved using skeletal muscle as a model. We will review recent advances about the differentiation, activation, and subtype specification of circulating monocyte to resolution or repair-type macrophages during the process we term regenerative inflammation, resulting in complete restoration of skeletal muscle in murine models of toxin-induced injury.

Keywords

Macrophage; Tissue repair; Muscle Regeneration; Regenerative inflammation; Acute; Sterile injury; Macrophage subtype specification; Myeloid cells; Monocytes

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1. The evolutionary and developmental relationship between immunity and regeneration

The extraordinary ability of anamniotes, amphibians, and fish to regenerate various body parts and entire organs such as the tail, limbs, spinal cord, intestine, eyes, heart, etc. is in very stark contrast with the loss of organ regeneration in amniotes, reptiles, birds and mammals [34, 48]. One might wonder why higher vertebrates lost their ability to regrow limbs or ears. In order to understand the critical but evolutionarily changing relationship between the immune system and an organism's ability to regenerate, it is worthwhile to have an overview of some of the branches of the evolutionary tree and the life cycle of certain highly regenerative animals. In certain vertebrates, if an organ is injured in postembryonic life, regeneration allows the complete rebuilding of the same organ using the blueprint of embryonic morphogenesis [36,64, 94]. These vertebrates, typically certain anamniotes, whose life is adapted to the aquatic environment, in particular amphibians with the capacity to regenerate extensively entire organs [112,23,79]. One school of thought posits that amphibian regeneration is linked to three things: (1) the transition from water to land via the larval stage and thus having elaborate genetic programs for metamorphosis in which the immune system recognize antigens of larval tissues as not-self and destroys the transitory tadpole organs that are replaced by new organs [5], (2) regeneration is facilitated by a high level of hydration and hyaluronate content to allow the formation of the blastema [4] and (3) an underdeveloped or suppressed immune system to allow embryoid tissue to develop and new structures be formed. Thus, the low activity or underdeveloped nature of the immune system, in particular the adaptive arm of it, in anamniotes facilitates organ regeneration since the blastema formed after an injury is tolerated long enough to allow regeneration [44–46,62]. These characteristics are lost in terrestrial vertebrates, with one exception, the lizards, which are able to regrow their tails thanks to immunosuppression [125,124] and the unique process called autotomy [3]. The rest of the vertebrates lost large parts of their genomes responsible for instructing the organized destruction and rebuilding during metamorphosis, developed a much more sophisticated immune system prohibiting embryonic tissue growth, fight microbial invasions and the consequences of UV irradiation and thus injury results in scars and fibrotic tissue formation than *ad integrum* regeneration [125,124]. Importantly, the limited ability to regenerate in amniotes requires the active contribution of the immune system, in particular its most ancient component, the phagocyte. These types of cells contribute to both the destruction and elimination of the injured or degenerated tissue and its rebuilding. Due to the fact that this process represents a rare example of full regeneration in mammals understanding its mechanistic components (inducing signals, regulators, and effectors) can be instructive in finding new ways to treat degenerative diseases. Next, we will review our current understanding of how this takes place in skeletal muscle.

The development of skeletal muscle fibers in mammals recapitulates this intriguing relationship between stem cell activity and inflammation. Skeletal muscle growth during the early phases of postnatal development (from P0 to P21 in the mouse) is dependent on and accompanied by a continuous increase of myonuclei leading to satellite cell fusion [82, 129]. Muscle regeneration following injury recapitulates most aspects of embryonic and

neonatal myogenesis, with satellite cells providing the major myogenic stem cell pool and undergoing proliferation and fusion, resulting in new myofibers [20]. A very distinct feature of muscle regeneration is though, which is absent in normal muscle development, is the key and coordinating role of inflammation and distinct macrophage populations required for the muscle growth process. On the other end of the developmental spectrum, during aging, the body's ability to regenerate its tissues, including skeletal muscle, is declining [10,106,92].

2. Phases of necrosis-induced tissue repair

Tissue repair is a vital process that allows the replacement of acutely damaged and necrotic cells and the restoration of organ function in mammals such as mice and also in humans [132]. When tissues are injured, the immune system acts to contain potential threats and restore tissue function and integrity [72]. Tissue injury can be caused by infections, exposure to toxic compounds, burns, and acute trauma, and while the immune events might vary depending on the injury, the phases leading to tissue recovery are relatively conserved. These phases are pro-inflammatory, resolution, tissue repair, and return to tissue homeostasis and heavily depend on the activity of the innate part of the immune system (Fig. 1) [127,134,14,6,72,86]. Impaired injury-related immune response has been shown to greatly influence regeneration in several tissues such as the liver, central nervous system, or skeletal muscle [14,32,68,95]. Importantly, immune cells and, in particular, monocyte-derived macrophages have a dual role during damage and regeneration [15,113]. First, these cells need to react to the injury, remove necrotic debris and in the regenerative inflammation phase, initiate restoration of tissue integrity via promoting resolution of inflammation and repair mechanisms.

Initially, acute injury or trauma results in tissue areas characterized by necrotic cell death mainly due to loss of plasma membrane integrity. Subsequently, the innate immune system is activated by the release of intracellular contents and molecules that are regarded as endogenous danger signals because they can trigger potent inflammatory responses (Fig. 2). Such molecules are referred to as alarmins or "Damage-Associated Molecular Patterns" (DAMPs). They can originate from different sources and include proteins of extracellular matrix (such as biglycan, versican, and heparan sulfate), intracellular proteins (such as histones, high-mobility group box 1 (HMGB1), S100 proteins and heat-shock proteins (HSPs)), and plasma proteins (such as syndecans and glypicans) [47]. In addition to intra-cellular molecules, intracellular stores of biologically active metabolites such as heme (released from myoglobin), lipids/lipoproteins and pro-inflammatory cytokines and chemokines, such as IL-1 α and IL-33 may also be released by damaged and necrotic cells [103,17,21,22,33,78,80,81,9,93]. Although these factors are not prototypical alarmins, they can mediate sterile inflammatory responses. When the tissue damage is limited, muscle resident immune cells (i.e., macrophages, dendritic cells (DCs), mast cells, CD8+ T cells, Tregs) perform their stereotypic maintenance function to clear the necrotic cells and establish immune tolerance [86,87]. However, when tissue damage is too extensive to be handled by resident cells, they immediately recruit peripheral blood neutrophils, mast cells, and inflammatory monocytes that predominately constitute the first wave of immune cell influx [15,113]. This initial activation will result in the production of inflammatory mediators, such as lipid mediators (e.g., prostaglandins, leukotrienes),

histamine, interleukins (e.g., IL-1 and IL-33), and other cytokines (OPN, IFN γ), and chemokines (e.g., macrophage migration inhibitory factor (MIF), CCL2, and CXCL8), which result in the recruitment of immune cells from the circulation to the affected area (Fig. 2) [137,62,66,89]. It is worth noting that in the case of open wounds (e.g., lacerations, trauma), the pro-inflammatory phase coincides with a clotting phase that is initiated by damage to the blood vessels and stops blood loss while protecting the wound from further exposure.

The second phase, described here as necrosis-induced inflammation, begins with infiltration of recruited neutrophils and monocytes to the injured area. These leukocytes phagocytose microorganisms, dying cells, and cell debris, thus preventing the spread of damage [26,136]. Eventually, the pro-inflammatory phase declines and progresses towards a regenerative inflammation phase, which starts with the resolution of inflammation followed by transition into a repair phase (Fig. 1). During regenerative inflammation, specialized pro-resolving lipid mediators (SPMs; Resolvin D2), anti-inflammatory and pro-repair cytokines (i.e., IL-10, TGF- β , AnxA1), and growth factors (i.e., GDF3, IGF-1) activate myoblasts and myofibroblasts (Fig. 2). This activation leads to the deposition of extracellular matrix (ECM) that serves as temporary protection, and at the same time, a scaffold for newly formed tissue. At this point, parenchymal cell growth is stimulated, which sets the beginning of re-establishing tissue structure and function and eventually the return to homeostasis. During this last phase, excess ECM is removed by macrophages and fibroblasts through the production of proteases such as cathepsins and matrix metalloproteinases (MMPs) [2]. The extent and duration of each phase are tissue-dependent and may be further influenced by external conditions and signaling factors [50].

As the inflammatory response triggered by sterile trauma or injury is analogous to that observed during microbial infection, host receptors that mediate the inflammatory responses to microorganisms may be involved in the activation of sterile inflammation [17]. Based on our current understanding, both Pathogen-associated Molecule Patterns (PAMPs) and DAMPs can similarly trigger inflammatory responses through the activation of classical Pathogen Recognition Receptors (PRRs), which include the Toll-like receptors (TLRs), the NOD-like receptors (NLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), C-type lectin receptors (CLRs) and several intracellular DNA sensors [17,97]. Interestingly, DAMPs can be sensed by a multitude of other receptors, which are referred to as non-PRR DAMP receptors, which include receptors for advanced glycation end products (RAGE), triggering receptors expressed on myeloid cells (TREM2), several G-protein-coupled receptors (GPCRs), scavenger receptors and ion channels [136,17,97].

Despite active research on this area, the mechanisms that uniquely define sterile and non-sterile inflammatory responses are still not well understood. There are still open questions (i) on how structurally diverse DAMPs signal to the few identified PRR DAMP receptors, (ii) whether there are more DAMP-sensing receptors to be identified, (iii) what are the interacting regions for DAMPs and their PRRs, and importantly, (iv) whether the dynamic cross-regulation and signal integration of PAMP- and DAMP-sensing receptors contribute to both host defense and sterile inflammatory diseases by recognizing PAMPs and DAMPs, respectively, and thus how any inhibition of DAMP-sensing receptor signaling during sterile

inflammatory diseases may also affect the risk to infection. Importantly, in support of this, it has been recently demonstrated that chronic infection prior to skeletal muscle injury impairs wound repair. Single-cell RNA-seq combined with flow cytometry analysis demonstrated that the preexisting inflammatory environment reduced the heterogeneity of the macrophage populations through the delayed transition of inflammatory to repair-type macrophages [58].

3. Mediator lipidome during acute sterile inflammation in skeletal muscle

Key determinants of the regenerative inflammation microenvironment are cytokines, growth factors, and lipid mediators, the so-called mediator lipidome. Lipid mediators play key roles in regulating both the initiation and resolution of acute inflammation. They are rapidly generated by immune cells and have direct receptor-mediated actions on immune cells, including neutrophils and macrophages [99,100]. Remodeling of membrane phospholipids occurs upon cellular activation as esterified polyunsaturated fatty acids (PUFA) are liberated for on-demand conversion to lipid mediators [30]. They are enzymatically generated from PUFA, such as arachidonic acid (AA), docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA), through the action of cyclooxygenases (COX), lipoxygenases (LOX), and cytochrome P₄₅₀ monooxygenases. These include pro-inflammatory eicosanoids such as leukotrienes (e.g., LTB₄, LTC₄) that regulate early microvascular permeability and leukocyte recruitment, and prostaglandins/thromboxane (e.g., PGE₂, TXB₂) that regulate blood vessel tone, pro-inflammatory signaling, and thrombosis [98]. In contrast, specialized pro-resolving lipid mediators (SPM), such as the resolvins (e.g., RvD1, RvD2, RvE1), are produced during the resolution phase of acute inflammation and actively signal the termination of pro-inflammatory cytokine and lipid mediator production, promote macrophage efferocytosis, and enhance host-defense [108,18,99]. SPMs are generated in humans, and recent studies have shown that they are produced in local inflammatory exudates (i.e., blisters) at bioactive levels and, when added back to human blisters, reduce neutrophil levels [100,83,96]. Informed by their endogenous roles in resolving inflammation, several studies have determined that exogenous delivery of SPM can improve pathological manifestations of chronic inflammation [27,107]. Moreover, recent evidence indicates that some SPM actively promote tissue repair in the skin and the eye and accelerate tissue regeneration in planaria [101,49,53].

We and others have also recently characterized the mediator lipidome in two murine acute muscle injury models (CTX and eccentric exercise-induced injury) during the transition from inflammation to resolution and regeneration in skeletal muscle injury and in exercised humans, using mass spectrometry-based lipidomics [122,42,77]. We observed the temporal regulation of glycerophospholipids and the production of pro-inflammatory (e.g., leukotrienes, prostaglandins) and SPMs (e.g., resolvins, lipoxins), which were modulated by COX enzyme inhibitors (i.e., ibuprofen). These time-dependent profiles were recapitulated in sorted neutrophils, and Ly6C^{high} and Ly6C^{low} muscle-infiltrating macrophages at various timepoints post-injury, with a distinct pro-resolving signature observed in Ly6C^{low} repair macrophages [42]. Given the emerging and diverse signaling roles of lipid mediators in controlling these distinct phases of the inflammation-resolution-repair response, future studies should aim to map the lipid mediator profiles during distinct stages of pathology (i.e., muscular dystrophy), elucidate specific lipid mediator signatures of innate immune cell

subsets, and integrate this information with available single cell and spatial transcriptomics for a systems-level comprehensive view of lipid mediator networks and immune cell phenotype. These key regulatory and effector mechanisms are intertwined and can be potentially exploited for therapies useful in diseases that have immune cell infiltration and/or defective regenerative capacity.

4. Macrophage subtype specification during tissue repair

Depending on their role and phenotype, infiltrating macrophages can be broadly classified as M1/M2, although in vivo studies suggest there is a spectrum of phenotypes and subtypes (Figs. 3A and 4) [40,54]. The widely accepted paradigm about the two main muscle-infiltrating macrophage populations posits that the initially appearing Ly6C^{high} macrophages are inflammatory, while Ly6C^{low} macrophages are reparative [119,120]. During the regeneration phase, Ly6C^{low} repair macrophages secrete cytokines and growth factors such as IGF-1, GDF3, IL-10, and TGF- β that act in a paracrine or autocrine manner and can significantly influence the repair cell milieu [115,121,29,35,73,92]. It can be assumed that during this latter phase, the immune response to tissue injury regulates the re-engagement of tissue progenitor cell populations to support cellular growth and differentiation. It is also likely that the microenvironment and reciprocal inter-cellular interactions mediated by autocrine and paracrine mechanisms are driving the inflammatory to repair phenotypic switch [91]. Our knowledge is still fragmented on how macrophages change their phenotype, employ sensory and regulatory mechanisms, and use effector functions to serve such reparatory roles. This is particularly important because the proper signaling between the participating cell types can ensure the precisely timed progression of repair while avoiding asynchrony, which can lead to delay, fibrosis, and chronic inflammation [24,114].

In order to gain insights into the dynamically changing immune milieu and immune cell phenotypes, we need to understand the sequence of events and molecular mechanisms of physiological regeneration. We and others have systematically profiled and identified regulatory and effector mechanisms of muscle-infiltrating macrophages using a sterile muscle injury model in mice injured by cardiotoxin [115, 121,29,73]. This model became a gold standard due to the facts that (i) it represents a robust, sterile inflammatory response, (ii) characterized by a highly synchronized and coordinated immune cell invasion, (iii) homogenous cell populations can be obtained easily, (iv) *ad integrum* regenerations is achieved without scarring and/or fibrosis and (v) it can be easily quantified using morphometric evaluation of the newly formed muscle fibers. Thus, it is well suited to explore the components and cellular and molecular interactions, including sensors, regulators, and effectors of regenerative inflammation. During the course of these studies, we evaluated the contribution of Ly6C^{high} (inflammatory) and Ly6C^{low} (patrolling) [41] circulating monocytes and found that inflammatory monocytes infiltrate the injured tissue first and these convert to repair-type macrophages later in situ [119]. This is an important advance because it argues that inflammatory (Ly6C^{high}) macrophages can be driven or reprogrammed to become repair type (Ly6C^{low}) cells [6,121]. We also generated a coherent and informative set of expression profiles on muscle-infiltrating inflammatory and repair macrophages from the CTX sterile injury model using cell sorting coupled to global

expression profiling in a time-course experiment (Fig. 3A) [121,42,93]. These results show the highly dynamic nature of the muscle macrophage response at the molecular level and document that a specific signature, primarily driven by the cellular milieu, is characterizing inflammatory and repair macrophages at each step of tissue injury and repair. These extended datasets have served as the basis for the prioritization of key transcriptional and effector pathways. Surprisingly, and despite their observed dynamic expression pattern in our datasets (Fig. 3B), molecules participating in critical inflammatory pathways like the inflammasome activation (NLRP3), negative regulation of TLR4 signaling (IRAK3), and AKT signaling (AKT1, AKT2) appear to be dispensable for proper muscle regeneration (Fig. 3C–3E) [111,123, 7,74]. These findings argue that initial macrophage activation has evolved with a large degree of redundancy to safeguard against uncontrolled or inadequate activation.

Monocytes are common progenitors of infiltrating macrophages and DCs. However, there is some evidence that they can influence wound-healing processes while maintaining their monocytic phenotype [25]. There are studies suggesting that monocytes are able to move through tissues while maintaining their monocytic phenotype without differentiating to macrophages or DCs [57]. Early studies suggested that monocytes infiltrate the injured tissue in two waves: the first wave involves an influx of classical Ly6C^{high} CCR2⁺ monocytes contributing to inflammation and angiogenesis followed by a later influx of non-classical (patrolling) Ly6C^{low} CX3CR1⁺ monocytes that contribute to scar formation [56,131]. However, in the context of muscle injury, we have shown monocytes themselves can restore homeostasis after tissue injury by differentiating into macrophages [119]. We showed that classical Ly6C^{high} monocytes that infiltrated mouse muscle tissue after a sterile injury changed their phenotype in situ towards non-classical Ly6C^{low} CX3CR1⁺ monocytes/macrophages. This change was IL-10 but not IL-4 dependent and associated with a decrease in dead cells and tissue debris. This suggests that classical monocytes are capable of re-establishing homeostasis of tissues by relying on in situ differentiation to inflammatory and later to pro-repair macrophages. It is possible, however, that the resolution of injury observed in this study also had a contribution from local macrophages. It is not known whether this classical-to-non-classical monocyte differentiation in situ occurs in other tissues and with other types of injury. These observations may challenge the idea of some researchers to refer to infiltrating tissue monocytes as macrophages and reflect the possible influence of monocytes on the fate of a tissue after injury.

The next-generation sequencing (NGS) technology's rapid progress provides a unique opportunity to study and deconvolute the cell milieu of the complex muscle regeneration process. A recent publication used droplet-based single-cell RNA-sequencing analysis on four time points post noxine-induced muscle injury, including 0 (steady-state), 2, 5, and 7 days. The dynamics of major immune cell populations were detected, and when compared with a healthy muscle, on Day 2, the presence and percentage of immune cells increased from 5% to 84%. In addition, the overall cell type composition shifted from myeloblasts (Cd79, Ly6d) and APCs (Ly22, Cd74, Ccl6) to pro-inflammatory macrophages (Ccl9, Ccr2, and Ly6c2). On day 5, the proportion of immune cells was still prevalent, but macrophage phenotypes shifted from pro-inflammatory to anti-inflammatory, with increased M2-like cell markers of C1q and Apoe. On day 7, immune cells started to present features

observed in the uninjured state [28]. These experiments confirmed the existing knowledge about macrophages' function in regulating the skeletal muscle regeneration process at the single-cell level. In parallel, Oprescu et al. performed scRNAseq but with even more time points following acute skeletal muscle injury (including 0.5, 2, 3.5, and 5, 10, 21 days post-injury). Similarly to the previous study of uninjured muscles, immune cells made up 7% (Cd3⁺/Cd4⁺ T cells, DCs, monocytes, and neutrophils) of the total cells. Immediately after injury, immune cells accounted for 87% (Vcan, Cxcl3, and Chil3 leukocytes, Cd36, Arg1, Spp1 Fabp4, and Fabp5 M1 macrophages, and S100a8/S100a9 neutrophils). At days 3.5 and 5 post-injury, Il7r⁺ macrophages (Gpnmb, Msrb1, and Pld3), M2-like macrophages (C1qa, C1qb, C1qc, Ms4a6, and Ms4a7), and Ly6C⁺ monocytes (Cd52, Ccr2, and Tlr2) were detected, but the number of immune cells began to decline by day 5 as expected. However, according to this dataset, immune cells comprised the largest population and displayed the most dynamic, transient, and time-dependent transcriptional features than other cell populations even at later stages (day 5 and 10 post-injury), including an Mrc1⁺ M2-like macrophage with antigen-presenting capacity (H2-Aa, H2-Eb1, and H2-Ab1) [88]. Collectively, these recent scRNA-seq datasets clearly demonstrate that in response to muscle injury, the immune system is phenotypically shifting gradually from a pro-inflammation stage to a regenerative inflammation stage to promote resolution and repair, which is confirmed by the specific expression of known anti-inflammatory and repair markers. These findings suggest that the immune cell lineages may also be interpreted as a hierarchical continuum of cell states (Fig. 4). However, it remains to be resolved how global profiles in cell cycle mediators, regulatory factors, and surface markers define this monocytic/macrophage continuum.

5. Macrophages promote debris clearance and resolution through interactions with neutrophils

Neutrophils are the most abundant leukocytes in the circulation and are the first responders to arrive at an injured tissue. Kolaczowska, Kubers [65,133]. In addition to their non-sterile antimicrobial function, neutrophils can phagocytose necrotic debris and modulate the inflammatory milieu by recruiting more neutrophils and monocytes to the injury site through the production of lipid mediators and chemokines [130,42,65]. It is hypothesized that shortly after accomplishing their function in injured tissues, neutrophils would die by apoptosis and would be phagocytosed by infiltrating macrophages [12]. This event may promote the phenotypic shift of macrophages towards an M2-like phenotype with increased anti-inflammatory cytokine production like IL-10 and decreased pro-inflammatory production (i.e., IL-12 and IL-23), thus promoting tissue repair [37]. Uncontrolled activation of neutrophils can potentially impede the healing process, which could lead to pathology [133]. Lastly, some tumor-related studies have suggested neutrophils themselves can acquire pro-inflammatory/anti-inflammatory profiles (N1/N2) [39]. However, the implications or existence of such phenotypes in tissue repair remain elusive. These interesting discoveries show that neutrophils may be more important than usually considered, and therefore it might be of interest for future studies to focus on the role of this cell type in the later stages of regenerative inflammation.

6. Macrophages promote regeneration through reciprocal communication with satellite cells

Muscle satellite cells play a crucial role in skeletal muscle regeneration. In 1994, the first study of macrophages' function in regulating myogenesis using in vitro culture of satellite cells with macrophage-conditioned medium showed enhanced myoblast proliferation activity [75,76]. Following skeletal muscle injury, activated satellite cells, once released from their stem cell niche, will directly interact with the stromal cell components, mainly composed of monocytes/macrophages, specifically through muscle progenitor secreted chemokines. The recruitment of monocytes and interaction of satellite cells with macrophages is reciprocal and indispensable for proper muscle regeneration, as demonstrated by an observed delay in the macrophage phenotype transition to Ly6C^{low} repair macrophages in the regenerating muscle of mice that were depleted of satellite cells (by irradiation) prior to injury [16,91].

As macrophages have multiple dynamic subsets during the muscle regeneration process, and to narrow down individual cell populations' function in regulating myogenesis, Chazaud's lab has demonstrated that the Ly6C^{high} pro-inflammatory macrophages could secrete pro-inflammatory cytokines, thus promote satellite cell activation and proliferation and repress differentiation. However, the Ly6C^{low} anti-inflammatory macrophages could promote myoblasts differentiation and suppress their proliferation through anti-inflammatory cytokines [6], even though the exact combination of these paracrine factors remains unclear to this day. There are many factors secreted by macrophages that have been shown in regulating this process, including IL-6 and TNF- α , both secreted by Ly6C^{high} macrophages to activate satellite cells following muscle injury [135]. In addition, Adamts1 is also induced explicitly in the Ly6C^{high} macrophages after injury. As a critical ECM regulator, Adamts1 works to repress Notch1 expression and thus decrease the Notch signaling pathway followed by increased activation of satellite cells during regeneration [31]. Moreover, macrophages contribute to the secretion of glutamine through increase glutamine synthetase activity and decreased glutamine oxidation to overcome the limitation of the intra-tissue levels of glutamine. In turn, uptake of glutamine by satellite cells stimulates mTOR signaling activity, which drives the activation of satellite cells and ultimately boosts muscle regeneration [102]. On the contrary, IL-4 [117], IGF-1 [105], and GDF3 [121] dominantly expressed by Ly6C^{low} macrophages, were demonstrated to promote myoblast differentiation, fusion, and muscle fiber growth.

A direct physical interaction between satellite cells and macrophages is another critical way to regulate mutual cell and regeneration activity [104,121,16,91]. Altogether, combining the data generated from both in vivo and in vitro studies, it has been demonstrated that both the temporal and spatial location of macrophage subpopulations can have complementary effects in regulating myogenesis during skeletal muscle regeneration. Importantly, satellite cells can also reciprocally promote and directly influence the acquisition of the different macrophage phenotypes [91].

7. Macrophages regulate muscle fibrosis by interacting with FAPs

Except from muscle satellite cells, Fibroadipogenic Progenitors (FAPs) are another critical stem cell type involved in skeletal muscle regeneration. They can be differentiated into fibroblasts or adipocytes and are a direct contributor to fat deposition and fibrotic scar formation [59,116]. In homeostatic skeletal muscle, FAPs are also one of the most prominent cell types. In the early phase of skeletal muscle injury, the number of FAPs increase dramatically, followed by a steady decline until they reach back to pre-damage levels [71]. During this process, FAPs contribute to satellite cell activation and proliferation. Conversely, satellite cells repress FAPs differentiation into adipocytes [84]. Nonetheless, FAPs could regulate the recruitment and promote the local expansion of the Foxp3⁺CD4⁺ regulatory T cell (Treg) population through the secretion of IL-33 and thus promote muscle regeneration [67]. IL-33 was also demonstrated to promote macrophage differentiation into the repair phenotype and activate myoblast differentiation [55]. Intriguingly, the decline of FAPs correlates with the peak of the infiltrating inflammatory macrophages accumulation. It was identified that the inflammatory macrophages could directly induce apoptosis of FAPs through the expression of TNF- α [71]. In contrast, TGF- β 1 secreted by the repair macrophages increases FAPs proliferation and ultimately leads to muscle fibrosis [109]. It was also reported that proteolytic cleavage enzymes of BMP1/MMP14 secreted by FAPs could transform the latent TGF- β into the activated version, which further acts on fibroblasts and promote the secretion of collagen [61]. Overall, macrophages play a crucial role in controlling fibrosis by regulating the survival of FAPs during skeletal muscle regeneration.

8. Macrophages regulate muscle angiogenesis through interacting with endothelial cells

Endothelial cells (ECs) regulate re-vascularization, which is a critical process for injured skeletal muscle to fully recover and return to homeostasis [1]. After an acute trauma, the number of ECs increases gradually and follows a similar pattern with the expansion of the capillaries close to the satellite cells [19]. A series of paracrine factors from ECs has been identified *in vitro* in regulating satellite cells activity, including angiopoietin-1 (Ang-1), IGF-1, hepatocyte growth factor (HGF), and vascular endothelial growth factor (VEGF), platelet-derived growth factors (PDGF) through co-culture experiments [11,19,8]. Macrophages play a central role in regulating their functions during skeletal muscle regeneration. CXCL12 mutant mice showed a delay in regeneration with persistent immune cell infiltration, aberrant fibro adipose deposition, and avascular defect formation [51]. Interestingly, depletion of monocyte infiltration in CCR2 mutant mice also shows impaired arteriogenesis [52]. It was further demonstrated that Apelin, Oncostatin M, and Periostin are molecular regulators of myogenesis/angiogenesis coupling. *In vitro* 3D triculture assays showed that anti-inflammatory macrophages could stimulate myogenesis and angiogenesis coupling during skeletal muscle regeneration in part through the secretion of Oncostatin M [69]. However, different macrophage populations' specific regulatory role in skeletal muscle revascularization is still controversial, and further studies need to be performed.

9. Macrophages interact with regulatory T-cells during muscle repair

As mentioned previously, following an acute injury, mononuclear myeloid cells infiltrate the damaged tissue, followed by a transition from pro-inflammatory to resolution/repair phenotype. Around the time of this phenotypic switch of the myeloid cell infiltrate, a Foxp3⁺CD4⁺ T regulatory cell population (Tregs) begins to accumulate -in markedly far fewer numbers though than myeloid cells- in the injured muscle, consisting half the total infiltrating CD4⁺ T cell population. Interestingly, macrophages and Treg cells spatially colocalized in the regenerating areas of injured muscle and experimental Treg ablation, either systemically or locally to the injured muscle, led to (i) an increase of immune cellular infiltrate, (ii) a failure of the myeloid cell compartment to undergo the expected phenotypic switch from pro-inflammatory to resolution/repair phenotype [13], (iii) a greater conversion of the MHCII to MHCII+ macrophages and (iii) increased IFN- γ production primarily by NK and effector T cells, which ultimately resulted in macrophage dysregulation, increased inflammation, and fibrosis, leading to impaired muscle repair [90].

10. Conclusions

Macrophages are positioned at the crossroads leading to acute inflammation, tissue repair, or regeneration. They participate in coordinating and linking the acute inflammatory response, the clearance of necrotic cells (i.e., dead parenchymal cells, polymorphonuclear neutrophils; PMN) during resolution to the promotion of tissue growth. Thus, these cells assume a spectrum of phenotypes and carry out first inflammatory functions and later tissue reparative roles [118,121,91]. This phenotype transition and its timing are critical in determining the physiological/functional and morphological/tissue architectural outcome of the injury, and it is regulated by endogenous microenvironmental cues. Several studies have shown that macrophages are required for tissue repair and regeneration. In acute skeletal muscle injury, depletion of macrophages by genetic or pharmacologic means impairs muscle regeneration and leads to fibrosis and deposition of adipocytes that impair muscle function [110,128,6,60]. A similar role of macrophages in tissue repair and regeneration has been observed in the heart, liver, and skin [118,38,63,70,85]. In humans, CD206⁺ macrophage numbers correlate with muscle growth following exercise [126]. Macrophage recruitment to skeletal muscle also facilitates angiogenesis [69]. While macrophages are required for tissue repair and regeneration, the prolonged presence of pro-inflammatory macrophages impairs tissue repair, while persisting tissue reparative macrophages leads to fibrosis [43]. This indicates that the chronic inflammatory environment specifically impairs the phenotypic transition from pro-inflammatory to pro-resolving/tissue reparative macrophage subtypes. The factors governing this impaired transition and whether this “halted” inflammatory phenotype can be resolved are still unclear. Altogether the evidence shows that myeloid cells are not only leading players in tissue repair but are also tightly associated with the development of pathological conditions such as fibrosis. However, the exact contribution and roles of each of these different macrophage subtypes of macrophages, as well as their cellular interactions, remains to be clarified. The recent development of various single-cell methods such as single-cell RNA-seq, ATAC-seq, CyTOF, and single-cell lipidomics, along with spatial proteomic and transcriptomic information, should greatly aid us in answering some of these questions.

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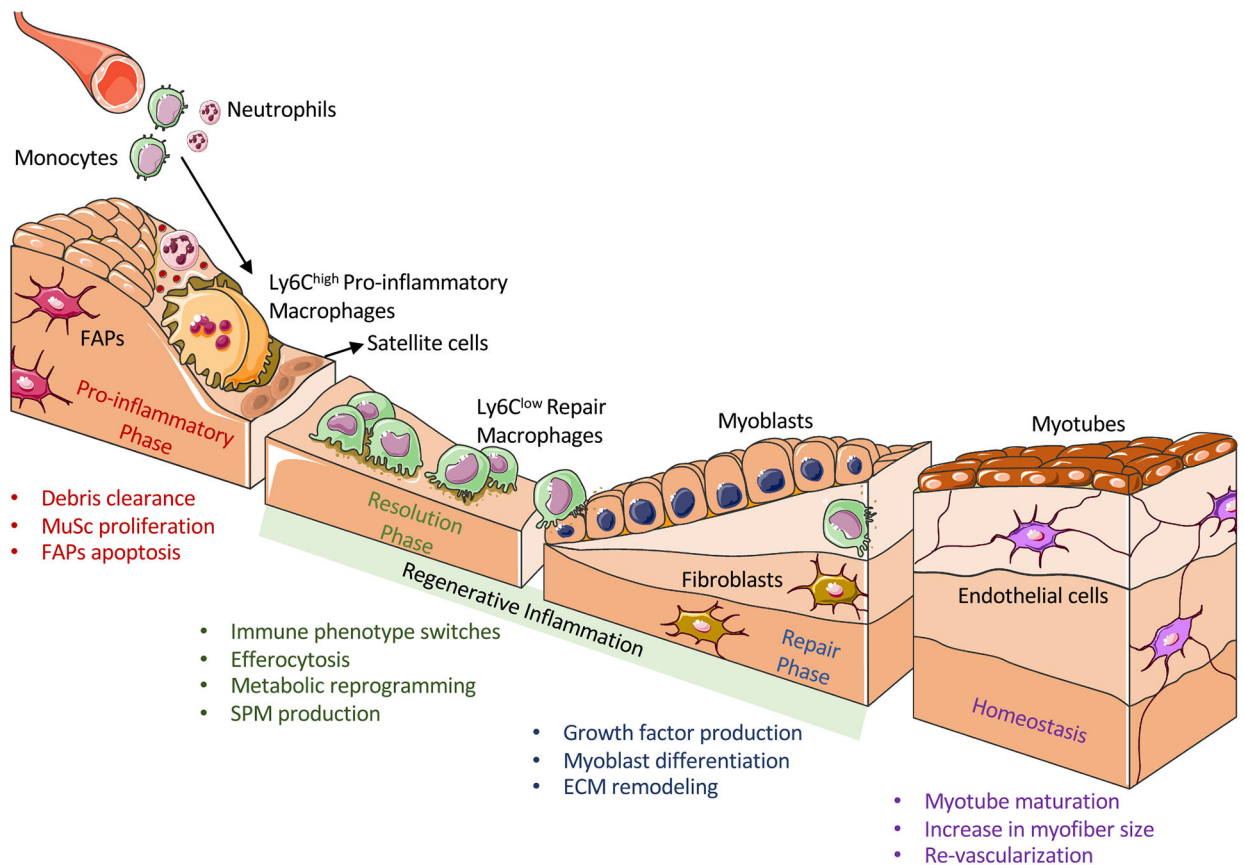


Fig. 1. Regenerative inflammation. Circulating neutrophils and Ly6C^{high} monocytes infiltrate early following acute sterile injury and give rise to Ly6C^{high} inflammatory macrophages to promote the clearance of necrotic debris. These cells stimulate MuSC proliferation and induce apoptosis of fibro-adipo-progenitors (FAPs) and fibroblasts. At the same time, muscle stem cells (MuSCs) exit quiescence and start to proliferate. Neutrophils typically undergo apoptosis shortly after. Integration of inflammatory lipid mediators (SPMs; specialized pro-resolving mediators) together with efferocytosis initiates the resolution of inflammation. The resolution phase is further supported by metabolic reprogramming of the Ly6C^{high} inflammatory macrophages. These events promote the phenotypic switch to a resolution/repairative macrophage phenotype. These Ly6C^{low} repair macrophages resolve inflammation while stimulating myoblasts, fibroblasts, and endothelial cells through the secretion of growth factors to promote cell cycle exit, fusion and myotube formation, re-vascularization, and ECM remodeling, respectively. The coordinated action and cellular interactions of macrophages with the injured cell milieu during regenerative inflammation are essential for efficient regeneration and the return to homeostasis.

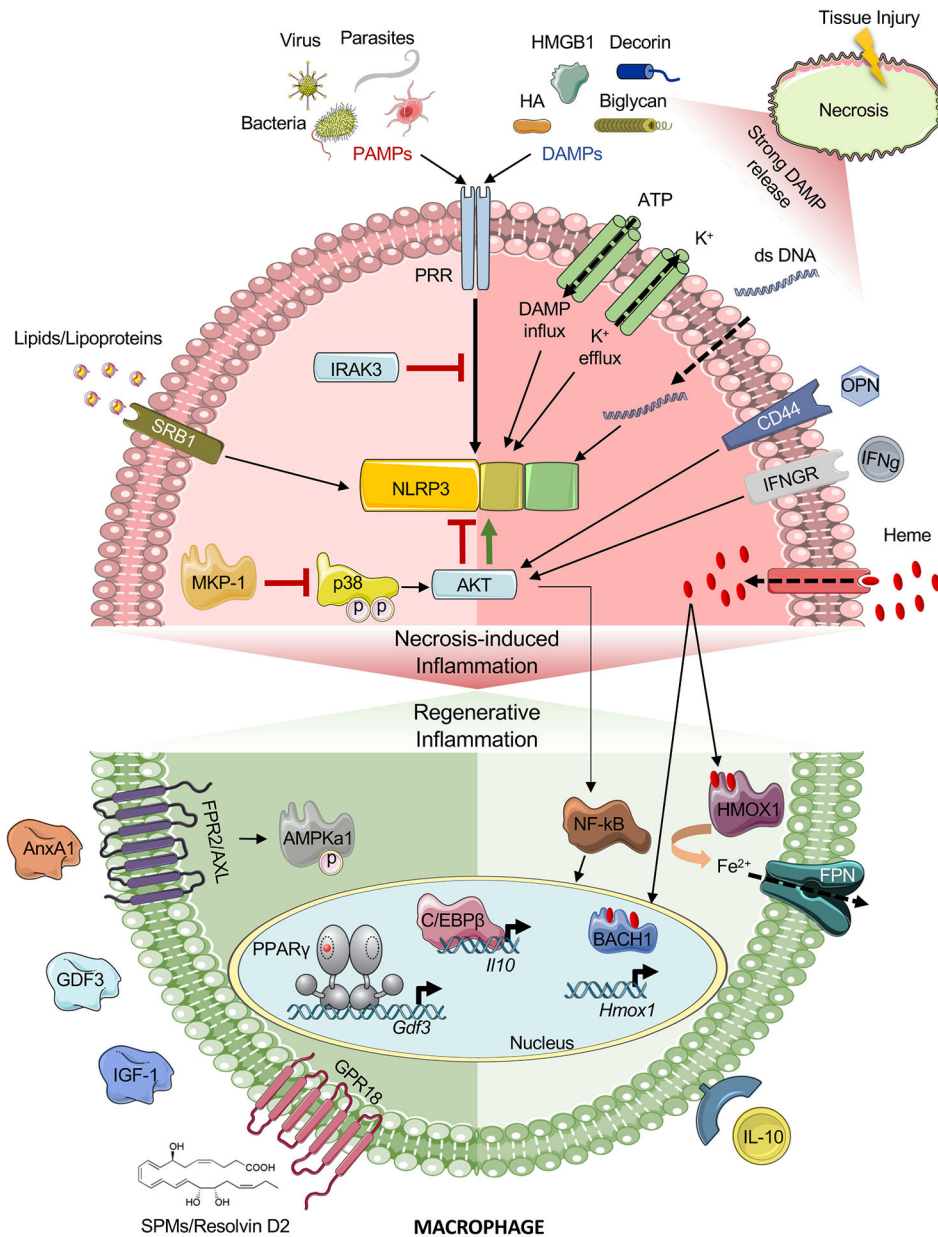


Fig. 2. Macrophage activation via Pattern Recognition Receptors (PRR) and autoantigens during regenerative inflammation. Schematic drawing depicting distinct ligand recognition and cellular location of pattern recognition receptors (PRRs) sensing various pathogen-associated (PAMPs) and damage-associated molecular patterns (DAMPs) during macrophage activation. Pathways involved in controlling macrophage phenotype and function during regenerative inflammation are summarized (AnxA1, AMPKα1, PPARγ-GDF3, IGF-1, C/EBPβ-IL-10, MKP-1, SRB1, BACH1-HMOX1, FPN, SPMs-Resolvin D2, OPN). Abbreviations: HMGB1, high-mobility group box-1; AKT, Akt serine/threonine kinase family; IRAK3, Interleukin 1 Receptor Associated Kinase 3; P2X7, P2X purinoceptor 7; NLRP3, NLR family, pyrin domain containing 3; TLR, toll-like receptor;

HA, Hyaluronic acid; ATP, Adenosine triphosphate; SRB1, Scavenger receptor class B type 1; OPN, osteopontin; MKP-1, Mitogen-Activated Protein Kinase Phosphatase 1; p38, p38 Mitogen-Activated Protein Kinase; IFNGR, Interferon-gamma receptor; CD44, Cluster of Differentiation 44; FPR2/AXL, Formyl peptide receptor 2; GDF3, Growth differentiation factor 3; IGF-1, insulin-like growth factor 1; IL-10, Interleukin-10; FPN, ferroportin; AnxA1, Annexin A1; AMPKa1, AMP-activated protein kinase; BACH1, BTB Domain And CNC Homolog 1; HMOX1, Heme Oxygenase 1; NF-kB, Nuclear factor-kappa B; GPR18, G Protein-Coupled Receptor 18; C/EBP β , CCAAT Enhancer Binding Protein Beta.

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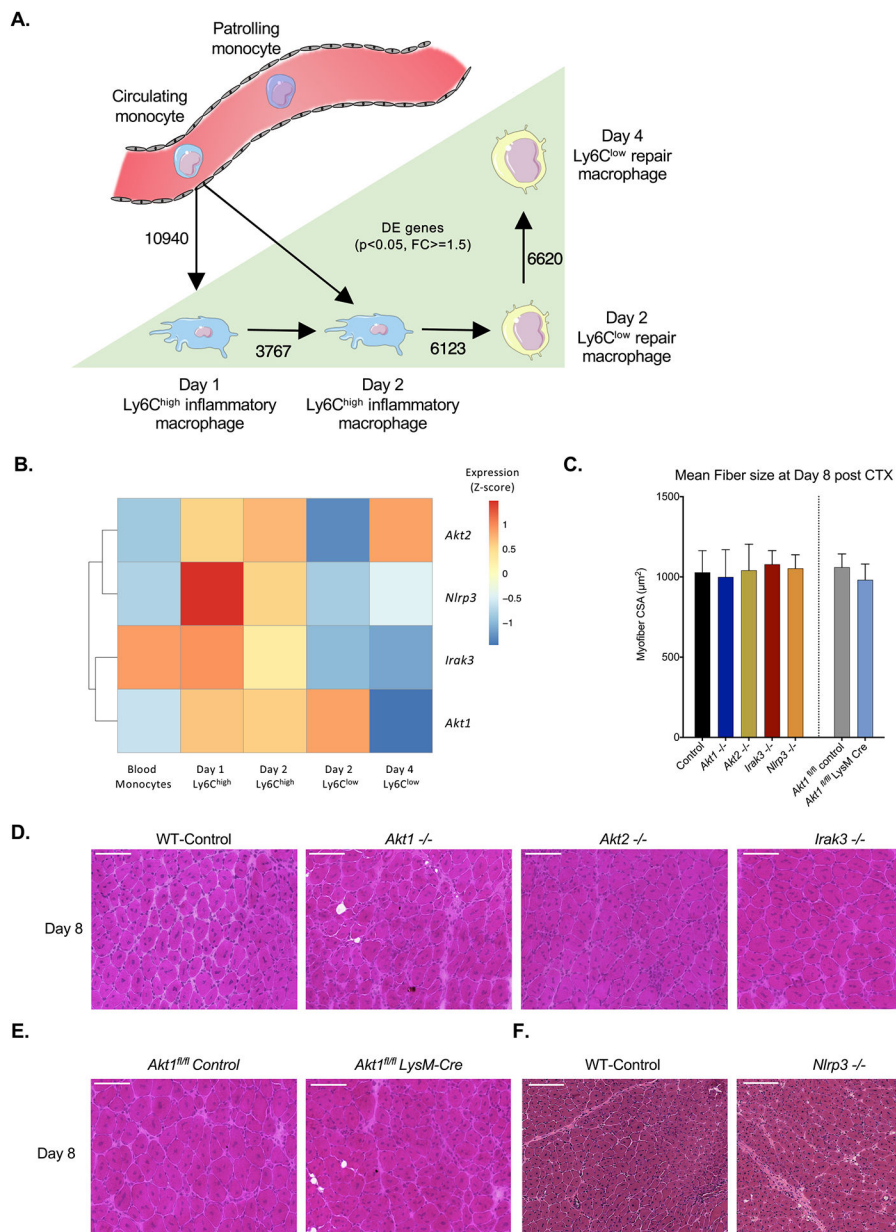


Fig. 3. Key inflammatory pathways are dispensable for proper muscle regeneration. **A.** Schematic depiction of differentially expressed genes ($p < 0.05$, $FC \geq 1.5$) during circulating monocyte to muscle-infiltrating inflammatory ($Ly6C^{high}$) and repair macrophage ($Ly6C^{low}$) transition. The number of genes changing is indicated per transition stage. Data are available under accession numbers GSE114291 and GSE164722. **B.** Heatmap representing the mRNA expression dynamics of key inflammation-associated genes in sorted blood monocyte, $Ly6C^{high}$ or $Ly6C^{low}$ muscle-infiltrating macrophages at indicated time points following cardiotoxin injury. Clustered RNA-seq expression values are visualized as Expression Z-score (calculated using the DEseq method). **C.** Average fiber CSA of regenerating tibialis anterior (TA) muscle in indicated mouse strains (8–10 weeks-old males) at day 8 post

cardiotoxin (CTX) injury (n = at least 4 mice per group). Bars and lines represent mean \pm SEM. D. Representative images of H&E-stained skeletal muscle (TA) from WT-control, *Akt1* KO, *Akt2* KO, and *Irak3* KO animals at day 8 post CTX-induced injury. Scale bars in the upper left corner represent 100 μ m. E. Representative images of H&E-stained skeletal muscle (TA) from *Akt1*^{fl/fl} control, *Akt1*^{fl/fl} LysM-Cre animals at day 8 post CTX-induced injury. Scale bars in the upper left corner represent 100 μ m. F. Representative images of H&E-stained skeletal muscle (TA) from WT-control and *Nlrp3* KO animals at day 8 post CTX-induced injury. *Nlrp3*^{-/-} mice were acquired through JAX (Stock #: 021302). Scale bars in the upper left corner represent 150 μ m.

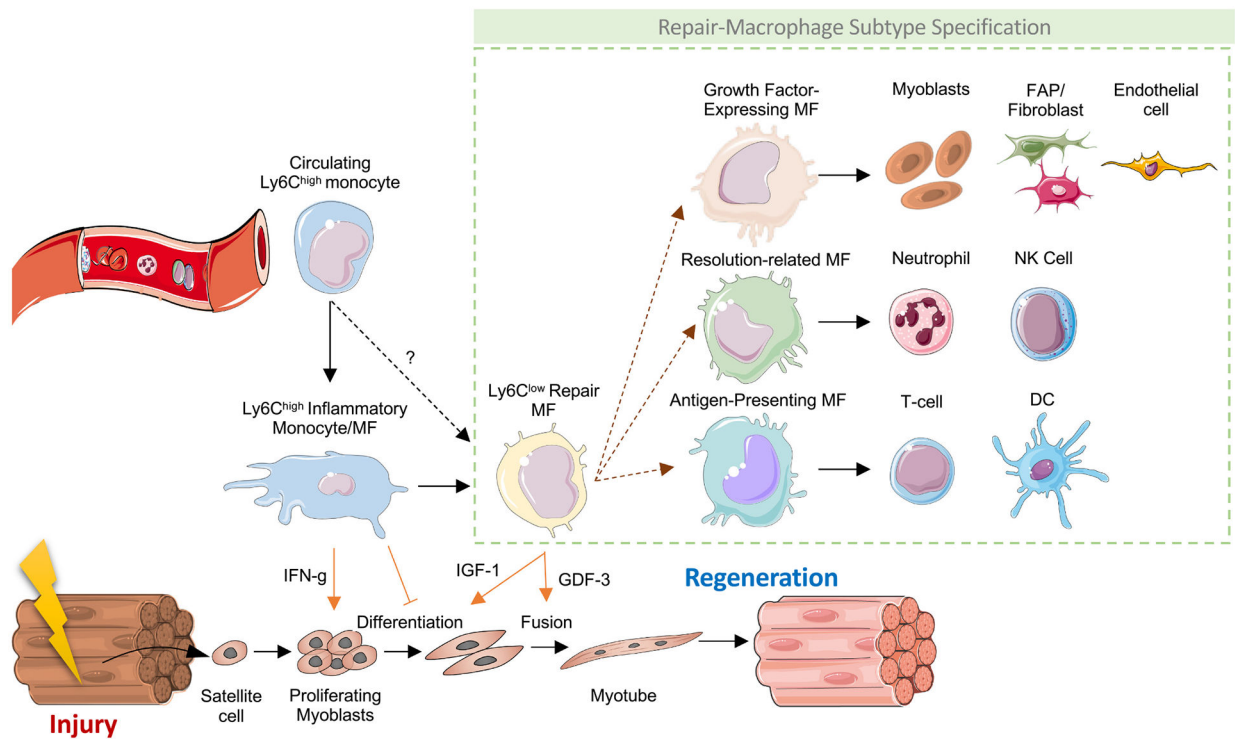


Fig. 4. Macrophage diversification and cellular interactions during regenerative inflammation. Schematic depiction of participating muscle-infiltrating macrophage subtypes and their cellular interactions during acute muscle injury and regeneration. During an acute sterile muscle injury, Ly6C^{high} monocytes extravasate from the circulation and start to phagocytose myofiber debris. These cells transition into Ly6C^{low} repair macrophages to promote wound healing through growth factor production. Recent advances in single-cell technologies propose the presence of multiple functionally distinct subtypes or states of repair macrophages that could preferentially influence the cellular interactions within the regenerating cell milieu. Possible subtypes of repair macrophages include Growth Factor Expressing, Resolution-related, and Antigen-Presenting macrophages.