Regenerative function of immune system: Modulation of muscle stem cells

Jasdeep Saini[1], Jamie S. McPhee[1], Sarah Al-Dabbagh[1], Claire E. Stewart[2] & Nasser Al-Shanti [1]*

[1] Work was performed in: Healthcare Science Research Institute, School of Healthcare Science Manchester Metropolitan University, John Dalton Building, Chester Street, M1 5GD, Manchester, UK.

[2] Present address: Research Institute for Sport & Exercise Sciences, School of Sport and Exercise Sciences, Tom Reilly Building, Byrom Street Campus, Liverpool John Moores University, Liverpool, L3 3AF.

* To whom correspondence should be addressed: Dr. NASSER AL-SHANTI. School of Healthcare Science, John Dalton Building, Chester Street, M1 5GD, Manchester, UK. tel: 0044 161 2475712; fax: 0044 0161 247 6831; email: n.al-shanti@mmu.ac.uk

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ABSTRACT

Ageing is characterised by progressive deterioration of physiological systems and the loss of skeletal muscle mass is one of the most recognisable, leading to muscle weakness and mobility impairments. This review highlights interactions between the immune system and skeletal muscle precursor cells (widely termed satellite cells or myoblasts) to influence satellite cell behaviour during muscle regeneration after injury, and outlines deficits associated with ageing. Resident neutrophils and macrophages in skeletal muscle become activated when muscle fibres are damaged via stimuli (e.g. contusions, strains, avulsions, hyperextensions, ruptures) and release high concentrations of cytokines, chemokines and growth factors into the microenvironment. These localised responses serve to attract additional immune cells which can reach in excess of $1x10^5$ immune cell/mm^3 of skeletal muscle in order to orchestrate the repair process. T-cells have a delayed response, reaching peak activation roughly 4 days after the initial damage. The cytokines and growth factors released by activated T-cells play a key role in muscle satellite cell proliferation and migration, although the precise mechanisms of these interactions remain unclear. T-cells in older people display limited ability to activate satellite cell proliferation and migration which is likely to contribute to insufficient muscle repair and, consequently, muscle wasting and weakness. If the factors released by T-cells to activate satellite cells can be identified, it may be possible to develop therapeutic agents to enhance muscle regeneration and reduce the impact of muscle wasting during ageing and disease.

Highlights:

- Immune cells infiltrate damaged skeletal muscles to release cytokines, chemokines and growth factors into the localised area that alter the micro-environment to clear cellular debris and activate muscle satellite cells.
- In young adults, the factors released by T-cells, in particular the regulatory T-cells, can extend the period of satellite cell proliferation to enhance muscle repair.
- In old adults, the T-cells do not release appropriate factors into the micro-environment and this may contribute to inadequate muscle recovery and consequently, to age-related deficits in muscle size and function.
- Identification of the factors released by young immune cells to regulate muscle regeneration could lead to the development of novel therapeutic agents to treat muscle wasting disorders and ageing.

CONTENTS

1. INTRODUCTION

1.1 Myogenesis and satellite cell activation

1.2 Young myogenesis

1.3 Aged myogenesis

2. INNATE IMMUNITY & MUSCLE REGENERATION

2.1 Innate immune response to acute damage and repair

2.2 Regulation of skeletal muscle regeneration via innate immune cell signalling

3. ADAPTIVE IMMUNITY & MUSCLE REGENERATION

3.1 Adaptive immune response to muscle damage

3.2 Regulation of skeletal muscle regeneration via Regulatory T-cells

4. CONCLUSIONS

5. REFERENCES

1. INTRODUCTION

In older age, skeletal muscle atrophies considerably (Maden-Wilkinson et al., 2014; Lexell et al., 1988; Janssen et al., 2002; Lexell, 1995; Morley et al., 2001), which contributes to weakness and mobility impairments inherent to sarcopenia (Janssen, 2011; Cruz-Jentoft et al., 2010) and frailty (Fried et al., 2001). Loss of skeletal muscle mass and function with ageing are associated with altered immune, hormonal and metabolic factors directly impacting on muscle (Narici & Maffulli, 2010) and resulting in motor unit remodelling (Piasecki, Ireland, Jones, et al., 2015; Piasecki, Ireland, Stashuk, et al., 2015). This review will first outline the role of the immune system in myogenesis that occurs after injury and then discuss how changes in immune cells may contribute to ageing-related muscle impairments. Identification of the signalling molecules exchanged between immune and satellite cells may lead to novel therapeutic strategies to preserve muscle with advancing old age and muscle wasting conditions.

1.1 Myogenesis and satellite cell activation

Skeletal muscle is the most abundant tissue type in healthy humans (Yin et al., 2013). It powers movements and contributes to metabolism by storing amino acids, glucose and fatty acids as well as oxidising substrates to replenish adenosine triphosphate stores (Leto & Saltiel, 2012). Muscles also release cytokines and growth factors into the extra-cellular compartments to act locally or systemically (Pedersen, 2011). The production of skeletal muscle cells occurs during embryonic myogenesis and thereafter myofibres themselves are incapable of proliferation (Bentzinger et al., 2012). Hence, the number of skeletal muscle fibres is largely determined before birth. Postnatal muscle growth arises by adapting and remodelling pre-existing fibres and through recruitment of resident, non-fused, self-renewing satellite cells (Tedesco et al., 2010). Satellite cells reside beneath the basal lamina of mature fibres in a quiescent state, they neither undergo cell division nor differentiation unless they are specifically activated to do so (Kuang et al., 2007). Damage to the muscle through injury or very intense prolonged unaccustomed exercise training are examples of principal activators of quiescent satellite cells.

1.2 Young myogenesis

Increases in muscle mass (hypertrophy) and adapted metabolism after exercise training in adults improves athletic performance and health (Egan & Zierath, 2013). The training-induced hypertrophy can depend on satellite cell proliferation and differentiation (Joanisse et al., 2013; Yin et al., 2013). However, hypertrophy may not necessarily require activation of satellite cells, since a satellite cell deficient mouse model showed normal training-induced hypertrophy (McCarthy et al., 2011; D. J. Glass, 2003). Satellite cells are, however, centrally involved in muscle regeneration after damage (Lepper et al., 2011; Yin et al., 2013). Some minor muscle damage can be a feature of everyday living that goes largely unnoticed by the individual due to minimal muscle tenderness and no apparent effect on function. More painful and functionally impairing damage can occur after repeated intense or rapid muscular activations, especially following unaccustomed high-load eccentric contractions (lengthening under strain) performed across a large range of motion (Paulsen et al., 2012) or electrical stimulation protocols (Crameri et al., 2007; Mackey et al., 2008; Nosaka et al., 2011). External stressors such as heavy impact causing contusion, traumatic puncture wounds or pathogen invasion can also damage otherwise healthy muscle, and in animal models, damage can be induced through injection of substances such as cardiotoxin (Ctx) (Sousa-Victor et al., 2014). Once activated, satellite cells migrate to the damaged site and re-enter into the cell cycle (Tedesco et al., 2010; Siegel et al., 2009) to generate the required concentration of myoblasts through several cycles of proliferation to regenerate damaged fibres. Although the majority of activated satellite cells differentiate into myotubes, a population of satellite cells return to a quiescent state (self-renewal) to maintain their numbers for the next incidence of muscle injury (Relaix & Zammit, 2012; Yin et al., 2013). The differentiated myotubes either fuse with pre-existing damaged myofibers to provide additional myonuclei during muscle regeneration, or fuse with each other forming de novo myofibers to replace the damaged myofibres during muscle regeneration (Adams, 2006; Siegel et al., 2011).

Satellite cells do not function in an isolated environment, a number of non-myogenic cells also populate muscle and influence the regenerative actions of satellite cells (Cerletti et al., 2008). For example, mesenchymal interstitial cells (Farup et al., 2015; Uezumi et al., 2014) and infiltrating

immune cells secrete numerous cytokines and growth factors into the localised microenvironment that orchestrate muscle regenerative mechanisms by clearing cellular debris and facilitating repair (Tedesco et al., 2010). These cytokines are not necessarily released into the general circulation to act systemically (Steensberg et al., 2002). Thus, effective muscle repair and regeneration relies not only on muscle satellite cells (known as the intrinsic niche) but also on other distinct cell types and their locally secreted cytokines (termed the extrinsic niche).

1.3 Aged Myogenesis

Muscle from aged mice was estimated to contain around 65% fewer functioning satellite cells than muscle from young mice (Cosgrove et al., 2014) and the overall number of satellite cells was also lower in aged mouse muscle (Chakkalakal et al., 2012). However, this was not the main cause of sarcopenia, at least not in mice, where induced depletion of satellite cells in young adults had little impact on the rate of muscle ageing (Fry et al., 2015). It is interesting to note that healthy older people do maintain the ability to activate satellite cells after intense exercise (Verdijk et al., 2009). However, if the activation of satellite cells cannot keep pace with damage, then muscle wasting or atrophy will inevitably occur. The loss of muscle mass with ageing has been linked to the reduced regenerative actions of older satellite cells and altered immune response to damage (Peake et al., 2010; Degens, 2010). There are reports of intrinsic deficiencies within satellite cells that reduce their activity. For instance, two-thirds of satellite cells in older mice showed reduced capacity for muscle regeneration due to elevated activity of p38α and p38β mitogen-activated kinase signalling which was not overcome by transplantation into a young recipient (Cosgrove et al., 2014). However, the debate continues as to whether or not satellite cell intrinsic deficits can be overcome by exposure to a ‘young’ microenvironment (reviewed elsewhere: (Brack & Munoz-Canoves, 2015)). There is strong evidence implicating the aged microenvironment with reduced satellite cell responses (Chakkalakal et al., 2012; Barberi et al., 2013). Transplanted muscle from young into old mice fails to regenerate, but transplanted muscle from old into young regenerate (B. M. Carlson & Faulkner, 1989), but might have a delayed regenerative response (Smythe et al., 2008). Moreover, ‘rejuvenating’ the microenvironment in older mice enhanced activation of

satellite cells through increased Notch signalling, as shown in heterochronic parabiosis models (Conboy et al., 2005; Morgan E. Carlson et al., 2008).

Lower satellite cell function with ageing was linked to increased activity of the transforming growth factor beta (TGF-β) family of molecules within satellite cells that are negative regulators of growth and restrict the proliferative responses (M. E. Carlson et al., 2009; Sousa-Victor et al., 2014; Yousef et al., 2015). Elevated fibroblast growth factor (FGF) signalling from the aged microenvironment was associated with depletion of the stem cell population and impaired regenerative capacity, but was countered in the aged satellite cells that had higher levels of Sprouty1 (Spry1) to inhibit FGF signalling (Chakkalakal et al., 2012). By altering satellite cell signalling through Notch, Wnt and receptor tyrosine kinases/extracellular signal-regulated kinase (RTK/ERK) it has been possible to overcome deficits in aged satellite cell function (Brack & Rando, 2007; Morgan E. Carlson et al., 2008; Naito et al., 2012). Circulating soluble factors, such as hormones, or other molecules released locally into the microenvironment may influence the intracellular satellite cell signalling to regulate proliferative and differentiation responses. For example, elevating the circulating oxytocin had rejuvenating effects for satellite cells (Elabd et al., 2014); increasing circulating levels of growth differentiation factor 11 (GDF-11) also rejuvenated satellite cells (Sinha et al., 2014). However, alternative research investigating the effect of GDF-11 on myogenesis observed a significant inhibition of skeletal muscle regeneration (Brun & Rudnicki, 2015). Additionally, elevated levels of osteopontin in aged mice was associated with impaired satellite cell responses to damage and this was overcome by reducing osteopontin *in vitro* and *in vivo* (Paliwal et al., 2012). Thus, a key detail, which has not yet been fully understood, is how the satellite cells respond to the rapidly changing microenvironment occurring soon after muscle damage, which is heavily influenced by the infiltrating immune cells.

2. INNATE IMMUNITY & MUSCLE REGENERATION

Changes in immune cells with ageing have been well characterised and the observations of elevated systemic inflammation led to the term ‘inflamm-ageing’ (Franceschi et al., 2000). Human immunity is subdivided into two main areas, often described as *innate* and *adaptive*

immunity. Innate immunity describes the primary capacity of the immune system to respond to pathophysiological triggers such as injury or pathogens and is mediated mainly through the myeloid progenitor cells (e.g. neutrophils, macrophages, dendritic cells, natural killer cells, mast cells, eosinophils, basophils) (Plackett et al., 2004). During normal physiological conditions, immune cells circulate within the blood and the lymphatic system, with considerable accumulations in lymphoid organs and most tissues of the body. Peripheral tissues also contain a population of resident immune cells, primarily consisting of macrophages and dendritic cells. However, during pathophysiological conditions supplementary leukocytes rapidly permeate tissues. During muscle regeneration, there can be in excess of $1x10^5$ immune cell/mm^3 of skeletal muscle (Wehling et al., 2001). When activated, these immune cells secrete cytokines and growth factors which regulate the damaged muscle microenvironment (Merly et al., 1999; Warren et al., 2004; Smith et al., 2008).

2.1 Innate Immune response to acute damage and repair

The regulation of infiltrating inflammatory cells is a dynamic process which varies depending on the extent of muscle damage and the time required to repair (Paulsen et al., 2012). Minor muscle damage, such as that which occurs after exercise, causes only a modest inflammatory response and may not cause substantial leukocyte cell infiltration, while more severe muscle damage occurring after very intense, unaccustomed exercise with high eccentric loads causes a considerably greater muscle tenderness, immune cell (e.g. neutrophil, macrophage and muscle T reg) infiltration (Fig. 1) of the damaged area and inflammatory responses consistent with rhabdomyolysis (reviewed in (Paulsen et al., 2012)).

The innate immune response to damage involves infiltration of inflammatory cells, but studies in aged mice have revealed a delayed inflammatory response (Shavlakadze et al., 2010). In healthy muscle, neutrophils show a transient response, infiltrating the extracellular space around the damaged fibres within 2 hours before concentrations decline to negligible levels within 3 or 4 days. The mechanisms of neutrophil infiltration remain unclear, but the resulting perpetuation of inflammatory damage is believed to be important for initiating the reparative process (Dumont

et al., 2008). Neutrophils release interleukin 1 (IL-1) and interleukin 8 (IL-8) which act as chemoattractants for macrophages, inducing the initial macrophage infiltration to the injury site (Fujishima et al., 1993; Cassatella, 1999). Resident macrophages within the endomysium and perimysium are also involved in phagocytosis and secrete enzymes, growth factors and cytokines/chemokines aiding the recruitment of additional immune cells (Wang et al., 2014).

Macrophages go through various stages of activation. *Classic activation* of macrophages is denoted as the M1 phenotype, where the increase in numbers and expression of proinflammatory mediators, cytokines and chemokines are observed from 24 hrs and reach peak activation around 2 or 3 days after damage (Rodriguez-Prados et al., 2010; Saclier et al., 2013) (Villalta et al., 2009). The M1 phenotype macrophages originating from the blood as monocytes are distinguishable by their expression of the glycoprotein lymphocyte antigen 6C (Ly6C) as well as receptors for the CX3C chemokine receptor 1 (CX3CR1) and C-C chemokine receptor type 2 (CCR2) (Geissmann et al., 2003). The chemokine CCR2 and its ligand CCL2 (or MCP-1) which are mainly produced by monocytes/macrophages coordinate the recruitment of macrophage $Ly6C^{+}$ to the site of injury supporting the proinflammatory response. $Ly6C^{+}$ monocytes differentiate into M1 macrophages in tissue and produce proinflammatory cytokines (Jetten et al., 2014). $Ly6C^{-}$ cells are recruited to the area by CX3CR1 and CCR2 chemokine receptor signalling and differentiate into M2 macrophages to perform anti-inflammatory and pro-myogenic functions that contribute to the later stages of regeneration (Forbes & Rosenthal, 2014). The M2 phenotype is known as *alternative activation* and peaks between 4 and 6 days (see Fig. 1) during the reparative process, where expression of anti-inflammatory mediators, cytokines and chemokines supports the regeneration through satellite cell activation (J. G. Tidball, 2005; Arnold et al., 2007). In cases of severe muscle damage causing fibre necrosis, macrophages can be found infiltrating the intracellular areas of fibres several days post-injury, and elevated macrophage concentrations are evident in muscle tissue up to 3 weeks later (Paulsen et al., 2010).

Fig. 1: Timeline of inflammatory responses and immune cells during regeneration.

CCR2$^{-/-}$ mice show impaired monocyte recruitment to the site of injury, while neutrophil and other T-cells remain unaffected (Abbadie et al., 2003). The CCR2$^{-/-}$ mice also show impaired muscle regeneration, arrested angiogenesis along with increased fibrosis and excess adipocyte accumulation at the injury site (Martinez et al., 2010). Bone marrow transplants from wild-type mice into CCR2$^{-/-}$ mice recovered the regenerative capacity of skeletal muscle of the CCR2$^{-/-}$ mice. These results show that CCR2, released by proliferating myocytes and resident immune cells, recruits bone marrow derived monocytes (Sun et al., 2009). However, the same results are not observed in studies involving CCL2$^{-/-}$ mice. The CCL2$^{-/-}$ mice have only a mild deficiency in regeneration, which may indicate that alternative chemokine (C-C motif) ligands can bind with the CCR2 receptor and support the recruitment of monocytes and ultimately improve regenerative capacity (Lu, Huang, Ransohoff, et al., 2011).

2.2 Regulation of skeletal muscle regeneration via innate immune cell signalling

In response to muscle injury, the innate immune system is activated, to enhance repair damaged tissue by secreting several cytokines (summarised in Fig. 2) (Madaro & Bouche, 2014). The cytokine interleukin 6 (IL-6) is involved in the initial infiltration of monocytes and macrophages during the inflammatory response shortly after muscle damage. Studies involving IL-6$^{-/-}$ mice revealed a significant decrease in the early infiltration of monocytes and macrophages to the injury site, resulting in diminished myofibre mass and more fibrosis of the muscle (Zhang et al., 2013). In the wild-type mice, much of the IL-6 produced soon after injury comes from the early monocyte and macrophage infiltration (Zhang et al., 2013). IL-6 also stimulates macrophage expression of another important molecule, granulocyte colony-stimulating factor (G-CSF), which is involved in normal myoblast proliferation and myofibre differentiation throughout the muscle regeneration process (Zhang et al., 2013; Wright et al., 2015). IL-6$^{-/-}$ mice show slower rates of hypertrophic muscle growth than wild-type animals (Serrano et al., 2008). This study also found that IL-6$^{-/-}$ animals have considerably lower levels of myogenin expression, but MyoD expression was unaffected, which helps to explain why myofibre differentiation was lower in IL-6$^{-/-}$ animals compared with wild-type.

Supplementary to IL-6 a rapid expression of tumour necrosis factor alpha (TNFα) after injury serves to intensify inflammation in the early stages following muscle damage and is linked to the innate immune response (Warren et al., 2002). TNFα is released by the resident neutrophils, along with interferon gamma (IFNγ) and Interleukin-1 beta (IL-1β), which can promote monocyte differentiation to M1 phenotype macrophages (Arango Duque & Descoteaux, 2014). Interestingly, as neutrophils and TNFα concentrations peak after 2 days post-injury, the quick tapering of neutrophils (3-4 days) is not paralleled by reductions in TNFα levels, which remain elevated for approximately 14 days after injury (Novak et al., 2014). This indicates that TNFα is not only involved with the early inflammatory process, but potentially has functions throughout muscle regeneration. Together, IL-6 and TNFα can enhance the proliferation of myoblasts, function as chemo-attractants aimed at myoblasts and immune cells, hinder the fusion of myocytes and affect development of stimulated satellite cells to the early phases of differentiation.

As mentioned earlier macrophages undergo various phases of activation. Specific cytokines such as the ones described (i.e. CCL2, IL- 6, and TNFα) are observed to be critically linked with classically activated M1 macrophage infiltration to the site of muscle damage through the initial inflammatory response. However, the differentiation of M2 phenotype macrophages is more complex than that of M1 (Mantovani et al., 2004). The Sub-phenotype M2a macrophages emerge from the exposure to cytokines secreted by adaptive immune responses, including interleukin 4 (IL-4) and interleukin 13 (IL-13), which stimulate the complex phases of tissue restoration and injury healing. The arrival of M2b macrophage are believed to begin with the provocation of Toll-like receptor immune complexes, leading to the release of anti-inflammatory chemokines such as IL-10 and the inflammatory cytokines TNFα and IFNγ (J. G. Tidball et al., 2014). TNFα can activate NF-κB within macrophages, which then induce the production and upregulation of additional proinflammatory mediators, including TNFα, which are then subsequently secreted by the macrophages into the microenvironment of the regenerating muscle. Research using $TNF\alpha^{-/-}$ mouse models showed a reduction in myogenic differentiation when compared to wild-type mice, this suggests that TNFα signalling within the immuno-muscular microenvironment

performs a regulatory role in muscle regeneration. (Chen et al., 2005). Alternatively, *in vitro* models using C2C12 murine myoblasts indicated that elevated TNFα hindered the myoblast capability to exit the cell cycle, indicating that TNFα prolonged myoblast proliferation while inhibiting myogenic differentiation.

TNFα can also activate NF-κB within myoblasts, resulting in myoblast proliferation through the up-regulation of cyclin D1 while suppressing differentiation, as well as inhibiting MyoD expression, further suppressing differentiation (Langen et al., 2004). Along with TNFα increasing proliferation and inhibiting differentiation through the NF-κB signalling pathway, NF-κB activation in myoblasts promotes the activation of p38 kinase. Animal studies have demonstrated that suppressing p38 leads to reductions in myotube formation along with lower levels of myogenin (Liu et al., 2012). When NF-κB signalling is activated within myoblasts via stimulation from TNFα secreted within the immuno-muscular microenvironment, an increase of IL-6 is also observed, delivering a supplementary route to enhancing the effects that NF-κB has on proliferation and increasing its suppression of differentiation. *In vitro* cell culture experiments where mouse myoblasts were treated with IL-6 displayed increases in myoblast proliferation, but not cell fusion (Pelosi et al., 2014). Likewise, *In vitro* cell culture experiments have shown that TNFα increase the migration capacity of myoblasts, demonstrating its role as a chemoattractant (Torrente et al., 2003). Providing further evidence that TNFα production by neutrophils and M1 phenotype macrophages following muscle damage promotes muscle regeneration via the attraction of satellite cells to the site of damage. Fig.2 shows the interaction of TNFα and the NF-κB signalling pathway and its influence on skeletal muscle regeneration.

The release of IL-10 by M2b macrophages also supports the recruitment of M2c macrophages, which release cytokines that are essential for the cessation of M1 macrophage infiltration and activity (J. G. Tidball et al., 2014). The IL-10 released by both M2b and M2c macrophages stimulates the proliferation of myoblasts needed for muscle growth and regeneration (Deng et al., 2012). Sub-phenotypes of M2b macrophages are observed throughout the repair process since their production of IL-10 is needed to promote anti-inflammatory actions during muscle

regeneration (Bosurgi et al., 2012). IL-$10^{-/-}$ mice show impaired transition of macrophages from the M1 to M2 phenotypes, resulting in a corresponding impairment to muscle regeneration. It is interesting to note that IL-$10^{-/-}$ mice are also used as an animal model of early-onset frailty with poor muscle mass and function in older age (Walston et al., 2008). Furthermore, mouse myoblast cell cultures supplemented with IL-10 and M2 macrophages resulted in enhanced myoblast proliferation (Deng et al., 2012). Therefore, IL-10 can mediate the transition of M1 to M2 macrophages after muscle damage occurs and encourages the proliferation of myoblasts and maturation of myofibers.

As myoblasts switch from proliferation to differentiation, a shift from M1 macrophages and proinflammatory cytokines to M2 macrophages and anti-inflammatory cytokines occurs concurrently. This cytokine transference diminishes the proinflammatory response and supports the differentiation of myofibres (Deng et al., 2012), thereby positively influencing the regenerative process (J. G. Tidball, 2005; Arnold et al., 2007). This is in part linked to insulin-like growth factor I (IGF-I), a protein known for its growth-promoting properties and anabolic-inducing effects through the up-regulation of myogenic regulatory factors (MRFs) (Chakravarthy et al., 2000; Mourkioti & Rosenthal, 2005; Xu & Wu, 2000). Importantly, IGF-I is also secreted by M2 macrophages during muscle regeneration (Tonkin et al., 2015; James G. Tidball & Welc, 2015). When observing the infiltration of monocytes and macrophages into the muscle injury site of $CCR2^{-/-}$ mice a considerable reduction of infiltrating cells is observed when compared to the controls. Interestingly, a reduction of circulating IGF-1 is also observed in conjunction with the reduced number of infiltrating immune cells. (Lu, Huang, Saederup, et al., 2011). This fascinating discovery indicates that macrophages provide growth factors that aid in the repair of muscle tissue damage by encouraging IGF-I stimulated satellite cell proliferation.

Overall, the regeneration of healthy young muscle occurs by rapid recruitment of immune cells to the damaged site in order to orchestrate the regenerative process by removing necrotic cellular debris, coordinating pro/anti-inflammatory events and activating satellite cells through strictly regulated signalling and chemo-attractant molecules (i.e. cytokines, chemokines).

Although damaged muscle fibres secrete a number of cytokines, chemokines and growth factors, it is the resident and infiltrating immune cells that are the main producers of these regenerating mediators. Consequently, any alterations to the numbers or types of cytokines, chemokines, or growth factors as a result of age related immune dysfunction has a considerable potential to disrupt the ability of satellite cells in elderly muscle to become activated, migrate to the site of injury, proliferate in adequate quantities and/or differentiate appropriately, resulting in an age linked decline of muscle size and function. Investigations regarding age associated changes to innate immune cell signalling molecules have discovered substantial difference when compared to young counterparts. Specifically, an increase in proinflammatory cytokines (i.e. IL-6, TNFα, IL-1β) is observed, leading to the chronic inflammatory state often observed in the elderly (Bruunsgaard et al., 2003; Ershler & Keller, 2000; O'Mahony et al., 1998). Increases in proinflammatory cytokines have been identified in the advancement of many geriatric disorders (Franceschi & Campisi, 2014). Thus, it can be appreciated that inflamm-ageing is also having a detrimental effect on the innate immune cells ability to properly coordinate the precisely programed stages of muscle regeneration, due to their inability to appropriately regulate the signalling molecules circulating within the immuno-muscular microenvironment during skeletal muscle regeneration.

Fig. 2: Innate immune signalling pathways in skeletal muscle regeneration.

3. ADAPTIVE IMMUNITY & MUSCLE REGENERATION

Adaptive immunity is observed as a secondary onset response to a pathophysiological incident, which is primarily mediated through lymphoid stem cells such as the T-cells and B-cells (Kim et al., 2007). There has been a remarkable increase in the number of descriptive studies detailing the interactions between innate immune responses and muscle regeneration. However, understanding of the role of the adaptive immune system in muscle regeneration is limited. Just as macrophages and cells involved with innate immunity are detected during acute muscle injury, adaptive immune cells such as T-cells are also present during the regeneration process (Cheng et al., 2008).

3.1 Adaptive immune response to muscle damage

T-cell infiltration to the site of injury is apparent approximately 3 days after injury and remains elevated for at least 10 days (Cheng et al., 2008). The satellite cells begin to migrate in damaged muscle in the initial 24 hours and begin to proliferate rapidly thereafter. These initial activities are likely regulated via cytokines secreted by innate immune cells (e.g. macrophages). However, adaptive immune responses to damaged muscle via the delayed release of cytokines by T-cells will promote continued satellite cell proliferation. The sustained T-cell presence throughout the regenerative process suggests that T-cells are fundamentally involved with skeletal muscle repair, but the mechanisms of these interactions are not well understood.

Experiments conducted with T-cell deficient mice resulted in a significant reduction in the early growth and development of muscle (Morrison et al., 2005). Cell culture investigations observing the impact of activated murine splenic T-cell cytokine secretions (secretome) on satellite cell function presented a ~24% increase in the proliferation of satellite cells isolated from young (3 months old) mouse muscle, compared to non-secretome treated satellite cell cultures (Dumke & Lees, 2011). Conversely, there was no significant effect on the proliferation of aged (32 months old) mouse muscle satellite cells when exposed to the same T-cell secretome. Furthermore, T-cells signalling (i.e. chemokines) also increased the rate of migration of young satellite cells but not old. However, T-cell secretome significantly reduced the ability of aged satellite cells to differentiate when compared to young satellite cells (Dumke & Lees, 2011). Additionally, recent research employing a mouse model observed that adding the secretome from human T-cells onto a punch-biopsy muscle wound accelerated healing (Mildner et al., 2013).

These findings reveal T-cell regulation of muscle repair, as well as the possibility that ageing may diminish T-cell regulated satellite cell function. Further research has explored the impact of T-cell secretome from activated and non-activated T-cells isolated from young (20-25 years old) human blood on immortalized murine satellite cells. The young activated-T-cell secretome enhanced proliferation of the satellite cells and reduced differentiation (Al-Shanti et al., 2014).

Demonstrating that regenerating muscle is influenced by, and responds to, a typical 'young' adaptive immune response. Follow-on work showed that the secretome from young (18-25 years old) activated T-cells enhances both proliferation and migration in immortalized murine satellite cell, however, the secretome from old (78-85 years old) activated T-cells induced premature differentiation similar to control conditions, with no effects on proliferation or migration of the satellite cells (Al-Dabbagh et al., 2015). This outcome implies that proteins secreted by the adaptive immune cells in young people enhance satellite cell proliferation and migration, whereas secreted proteins by the adaptive immune cells of old people attenuates satellite cell proliferation and migration by prematurely stimulating differentiation. These studies indicate that impairments in the ability of satellite cells in elderly people to appropriately proliferate and migrate to the site of muscle injury are related to age-associated T-cell deficiencies, promoting age-related reductions in skeletal muscle size and function.

Various studies have established that T-cells secrete growth factors and cytokines, some of which can influence satellite cell function (e.g. FGF2, IFNγ, TGFβ, TNFα, and IL4) (Blotnick et al., 1994; De Rosa et al., 2004; Levings et al., 2002). The challenge for future studies will be to determine how advanced ageing alters the specific types and concentrations of proteins secreted by old T-cells when compared to young T-cells. This will identify the up- and/or down- regulated immune factors responsible for altering satellite cell function during muscular regeneration in elderly people. Conceivably, these discoveries could lead to the manipulation of immune factors in the immuno-muscular microenvironment of elderly people, possibly replicating a young immuno-muscular microenvironment and overcoming the age associated defects in aged satellite cell function.

3.2 Regulation of skeletal muscle regeneration via Regulatory T-cells

Much of the early research beginning to expose the role of adaptive immunity on muscle regeneration has focused on investigating all T-cells as a single component of immunity interacting with satellite cells (Fig. 3). However, there are several different sub-phenotypes of T-cells and distinguishing between them during regeneration may be crucial for identifying which

T-cell sub- phenotypes are up and/or down regulating cytokines and growth factors that influence satellite cell function. Attention has been drawn to a specific population of immune response regulatory T-cells (Treg), denoted as the $CD4^{+}Foxp3^{+}$ sub-phenotype. Not only are these Treg cells involved with immune response regulation (Josefowicz et al., 2012), they have also been detected at concentrations of $1.05 \pm 0.38 \times 10^{4}$ cells/g of muscle 28 days after injury. However, alternate T-cell sub-phenotype populations decrease to pre-injury levels of $0.13 \pm 0.06 \times 10^{4}$ cells/g of muscle by the same time point of the repair process (Dalia Burzyn et al., 2013). This finding indicates that Treg cells may be a vital immune cell type influencing muscle regeneration.

Using mouse models with muscular injury induced via Ctx, it was shown that the Treg cell concentrations increased within the injured muscle as the innate immune cells shifted from a pro- inflammatory to anti-inflammatory phenotype (i.e. M1 to M2) (Dalia Burzyn et al., 2013). It was also discovered that Treg cells found specifically in muscle (mTreg) produce distinctive proteins from their counterparts found in other tissues. These proteins include the anti-inflammatory cytokine IL-10 and the growth factors amphiregulin and platelet-derived growth factor (PDGF), all of which have been shown to influence typical muscle regeneration (Dalia Burzyn et al., 2013; Huey et al., 2008; Yablonkareuveni et al., 1990). Furthermore, experiments where Treg cells were prevented from entering the Ctx injured mouse muscle resulted in innate immune cells failing to switch from pro-inflammatory M1 phenotype to the anti-inflammatory M2 phenotype. Treg ablation from damaged muscle also caused and abnormal inflamed morphology of the regenerating muscle fibres with fibrosis (Castiglioni et al., 2015). Treg-stimulated satellite cells showed sustained proliferation and delayed differentiation (Castiglioni et al., 2015).

Although evidence has been presented outlining the role Treg cells perform during muscle regeneration, further research is required to fully understand how Treg cells are recruited and expanded within damaged muscle. It is also interesting to consider that Treg cells are able to influence muscle repair via interaction with innate immune cells (i.e. macrophages) as well as

activating satellite cells (D. Burzyn et al., 2013). These observations may help to serve as a foundation for future studies looking at the impact ageing has on Treg cells and whether ageing causes a reduction or increase in the number of Treg cells infiltrating the site of muscle damage. These studies may also help to determine if ageing impacts Treg cells' ability to produce the appropriate types and concentrations of cytokines and growth factors needed for normal muscle repair and regeneration.

4. CONCLUSIONS

Immune cell infiltration into the site of muscle damage and subsequent release of signalling molecules (i.e. cytokines and growth factors) into the microenvironment regulate muscle repair and regeneration through direct interaction with satellite cells (see Fig. 3). Immune factors released within an aged immuno-muscular microenvironment differ from those of young. Investigating specific populations and sub-phenotypes of both innate and adaptive immune cells, in both young and elderly people, will provide insight into the mechanisms of age-associated muscle wasting. Developing novel therapies to treat sarcopenia by manipulating the aged immuno-muscular microenvironment during regeneration may enhanced muscle size and restore muscle function in the elderly. Current strategies to promote muscle regeneration and maintenance in elderly people are primarily focused on nutrition and physical activity (English & Paddon-Jones, 2010; Moore, 2014). These approaches may alleviate the progression and trajectory of sarcopenia, but only to a relatively minor degree. These therapies are only able to delay the inevitable loss of skeletal muscle mass, function and regenerative capacity associated with progressive ageing. A number of pharmacological strategies to tackle muscle wasting have been proposed, although no treatments are currently in clinical use that block or reverse the loss of muscle in the elderly (D. Glass & Roubenoff, 2010). Therefore, developing a novel approach to prevent sarcopenia is essential and elucidating the role of the immune system in muscle regeneration will help to identify regulatory processes that are candidates for intervention.

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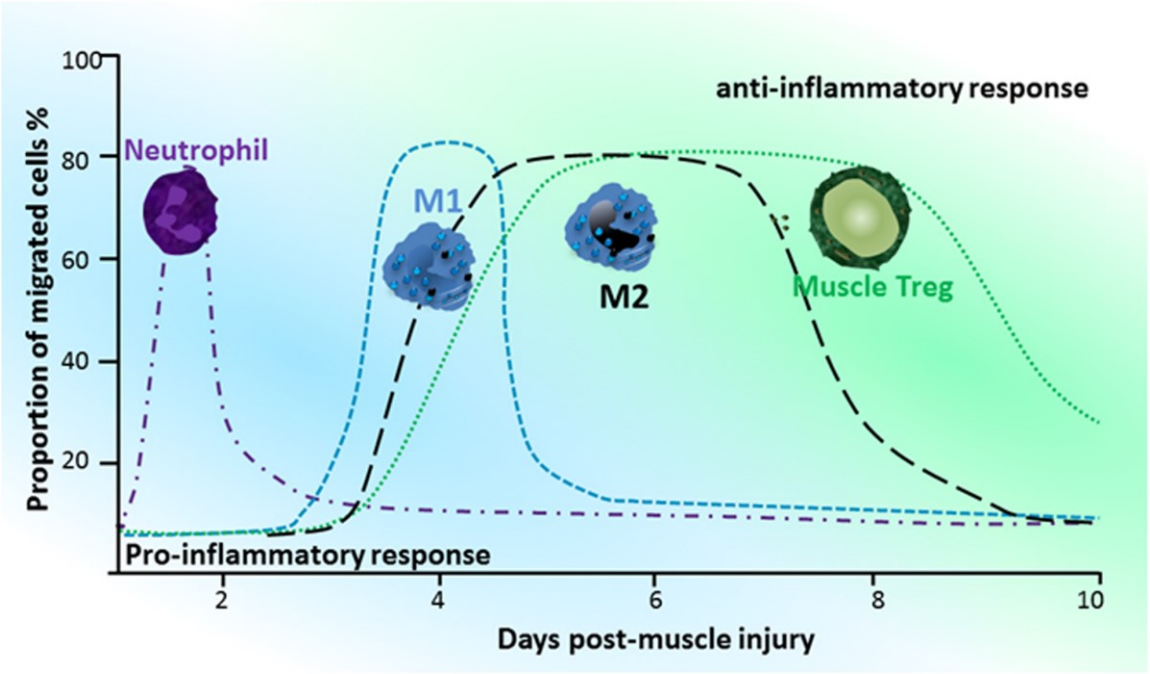

Fig. 1. Timeline of inflammatory responses and immune cell during muscle

The immune system responds to muscle damage by recruiting a precise sequence of pro and anti-inflammatory immune cells to the site of injury. Immune cells are observed from the initial pro-inflammatory phase required for removal of cellular debris through to the final repair of the damaged muscle fibres. Neutrophils rapidly infiltrate the extracellular space around the damaged fibres within 2 hours and peak in number between 6 and 24 hours followed by rapid decline of neutrophils to negligible levels within 72 to 96 hours. This initial infiltration of neutrophils further contributes to the inflammatory damage to the injured muscle fibres. Subsequently, M1 macrophage concentrations rapidly being to increase at the site of injury and initiate the pro-inflammatory functions of muscle repair through secretion of several cytokines and mediators. The number of M1 macrophages will continue to increase until peak concentrations at 72 to 96 hours after injury and then begin to decline sharply. This is followed by the increase in numbers of anti-inflammatory and pro-myogenic M2 macrophages, which reach peak concentrations in the regenerating muscle at roughly 120 to 144 hours post injury, remaining significantly elevated for several days following. Finally activated T-cells are recruited to the regenerating muscle damage site, with concentrations beginning to peak as M2 macrophage number being to decline. Populations of T cells, specify mTreg remain significantly elevated for 30 following the initial injury causing muscle damage. (Modified from **Tidball & Villalta, 2010; Forbes & Rosenthal, 2014**).

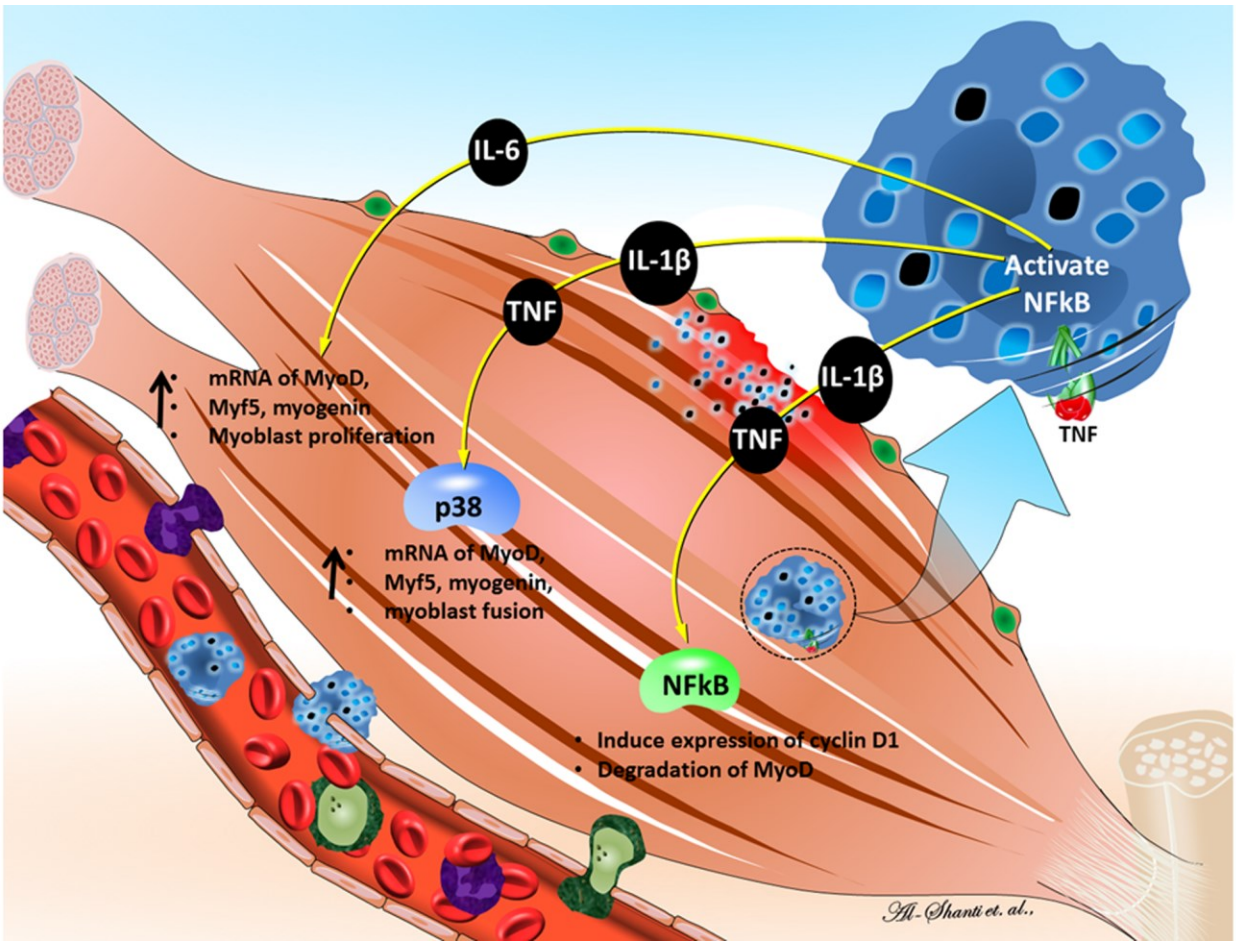

Fig. 2. Innate immune signalling pathways in skeletal muscle regeneration

The activation of NF-κB in either muscle cells or macrophages can affect muscle cell proliferation and differentiation. Cytokines (IL1β, TNFα and IFNγ) can increase NF-κB activation in both muscle and/or macrophages. The cytokines contribute to further activation of NF-κB in macrophages and muscle cells or they can act on the muscle cells themselves to affect their proliferation or differentiation. TNFα can activate NF-κB within macrophages, which then induces the production of additional proinflammatory mediators. NF-κB activation can promote proliferation of muscle cells through the up regulation of transcripts needed for cell cycle progression (cyclin D1), while suppressing differentiation by decreasing the expression or destabilizing transcripts needed for muscle to experience early and terminal differentiation (MyoD and myogenin). Along with TNFα promoting proliferation and inhibiting differentiation through the NF-κB signalling pathway, it can also promote later stages of differentiation through the activation of p38 kinase. Nuclear factor-kappa B (NF- κB), interferon gamma (IFNγ), Interleukin-1 beta (IL-1β), Tumour necrosis factor (TNFα), (Modified from **Tidball & Villalta, 2010, Pillon et al., 2013; Forbes & Rosenthal, 2014)**.

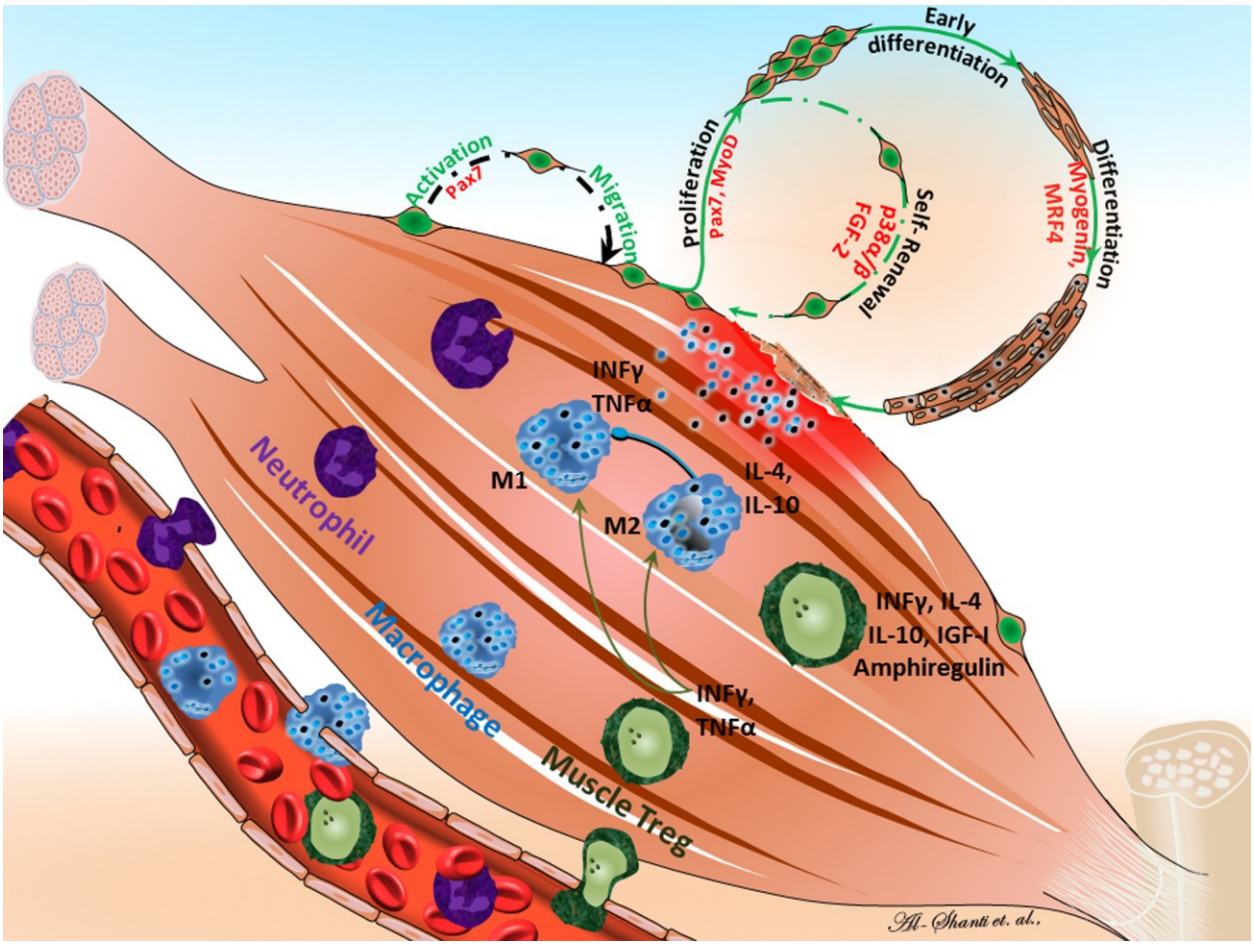

Fig. 3. Summary of general interactions between immune and muscle cells following acute muscle injury. Following muscle damage quiescent satellite cells become activated and begin to migrate to the site of injury. The satellite cells re-enter the cell cycle and begin to proliferate until a proliferative threshold is met. A required quantity of the proliferating satellite cells will self-renew to replenish the pool of quiescent cells while the remaining proliferating cells will continue to differentiate to repair the damaged muscle fibres. Importantly, the phases of satellite cell activation, migration, proliferation and differentiation are regulated by immune cells. The immune system responds to muscle damage with a complex sequence of reactions, which ultimately lead to inflammation followed by muscle regeneration. The initial infiltration of transient neutrophils contain and localize the damage in the muscle and clean up cellular debris. M1 macrophages secrete cytokines that induce satellite cell activation and proliferation. M2 macrophages that then promote muscle repair, differentiation and recruit T-cells to the injured muscle site. T-cells such as mTreg cells secreting numerous growth factors (e.g. IGF-I, amphiregulin) and cytokines, which may contribute to facilitating muscle regeneration. Insulin-like growth factor I (IGF-I), Interferon gamma (IFNγ), Interleukin-1 beta (IL-1β), Tumour necrosis factor (TNFα), fibroblast growth factor (FGF-2), muscle regulatory factors 4 (MRF4), Interleukin-4 (IL-4), Interleukin-10 (IL-10) (Modified from **Siegel, A. et al, 2009**; **Tidball & Villalta, 2010, Pillon et al., 2013; Forbes & Rosenthal, 2014)**.