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Mechanisms of Muscle Injury, Repair, and Regeneration

James G. Tidball[*1,2]

ABSTRACT

Skeletal muscle continuously adapts to changes in its mechanical environment through modifications in gene expression and protein stability that affect its physiological function and mass. However, mechanical stresses commonly exceed the parameters that induce adaptations, producing instead acute injury. Furthermore, the relatively superficial location of many muscles in the body leaves them further vulnerable to acute injuries by exposure to extreme temperatures, contusions, lacerations or toxins. In this article, the molecular, cellular, and mechanical factors that underlie muscle injury and the capacity of muscle to repair and regenerate are presented. Evidence shows that muscle injuries that are caused by eccentric contractions result from direct mechanical damage to myofibrils. However, muscle pathology following other acute injuries is largely attributable to damage to the muscle cell membrane. Many feaures in the injury-repair-regeneration cascade relate to the unregulated influx of calcium through membrane lesions, including: (i) activation of proteases and hydrolases that contribute muscle damage, (ii) activation of enzymes that drive the production of mitogens and motogens for muscle and immune cells involved in injury and repair, and (iii) enabling protein-protein interactions that promote membrane repair. Evidence is also presented to show that the myogenic program that is activated by acute muscle injury and the inflammatory process that follows are highly coordinated, with myeloid cells playing a central role in modulating repair and regeneration. The early-invading, proinflammatory M1 macrophages remove debris caused by injury and express Th1 cytokines that play key roles in regulating the proliferation, migration, and differentiation of satellite cells. The subsequent invasion by anti-inflammatory, M2 macrophages promotes tissue repair and attenuates inflammation. Although this system provides an effective mechanism for muscle repair and regeneration following acute injury, it is dysregulated in chronic injuries. In this article, the process of muscle injury, repair and regeneration that occurs in muscular dystrophy is used as an example of chronic muscle injury, to highlight similarities and differences between the injury and repair processes that occur in acutely and chronically injured muscle. © 2011 American Physiological Society. *Compr Physiol* 1:2029-2062, 2011.

Introduction

Skeletal muscle is an amazing tissue in many regards, and what it does, it does well. Most obviously, muscle is specialized to generate force but for that function to be well-served, muscle must be a precisely organized, excitable tissue. It also shows an impressive ability to adapt to changing functional demands and provides a major site for energy storage in the body. More subtly, muscle can serve an endocrine function, releasing factors and small molecules that can influence the growth, function, and development of other tissues. Because of the highly specialized functions of muscle, its high level of organization at the tissue, cellular, and molecular levels, and the large proportion of the body that comprises muscle, much of what we now know about the functions of other tissues was founded on studies first conducted on muscle. Muscle has been a "pioneering" tissue for understanding development and how transcription factors function to regulate tissue-specific gene expression. Muscle has been the tissue in which molecular motors were first explored and the relationship between changes in molecular structure and cell movements were first elucidated. Many of the premier studies of the mechanisms of electrochemical communication between cells were conducted on muscle. Many key studies concerning both aerobic and anaerobic metabolism have been performed on muscle. Furthermore, as noted by the great muscle physiologist Professor Douglas R. Wilkie, muscle is also "good to eat!" (Fig. 1).

Muscle has more recently provided a valuable system for studies directed at understanding how tissues respond to

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Available now. LINEAR MOTOR. Rugged and dependable: design optimized by world-wide field testing over an extended period. All models offer the economy of "fuel-cell" type energy conversion and will run on a wide range of commonly available fuels. Low stand-by power, but can be switched within miliseconds to as much as 1 KW mech/Kg (peak, dry). Modular construction and wide range of available subunits permit tailor-made solutions to otherwise intractable mechanical problems. Choice of two control systems:

1. Externally triggered mode. Versatile, general-purpose units. Digitally controlled by picojoule pulses. Despite low input energy level, very high signal-to-noise ratio. Energy amplification 10^6 approx. Mechanical characteristics: (1 cm modules) max. speed: optional between 0.1 and 100 mm / sec. Stress generated: 2 to 5 x 10^{-5} Newtons m^{-2}.

2. Autonomous mode with integral oscillators. Especially suitable for pumping applications. Modules available with frequency and mechanical impedance appropriate for:

a) Solids and slurries (0.01 – 1.0 Hz.)

b) Liquids (0.5 – 5 Hz): lifetime 2.6 x 10^9 operations (typ.) 3.6 x 10^9 (max.) – independent of frequency.

c) Gases (50 – 1000 Hz.)

Many optional extras, for example, built-in servo (length and velocity) where fine control is required. Direct piping of oxygen. Thermal generation. Etc.

Good to eat.

Figure 1 Lecture announcement for a presentation by Professor D.R. Wilkie given at the Institution of Electrical Engineers in London, UK on the subject of muscle physiology.

injuries caused by mechanical stress. More than any other tissue, muscle is obviously adaptive to mechanical stress, responding by changes in gene expression and modifications in protein stability and turn-over that can have profound effects on both the quantity and physiological attributes of the tissue. However, mechanical signals that can modulate muscle adaptation can also cause damage if the magnitude, duration, or frequency of application of the signals exceeds the capacity of muscle for load bearing. When muscle damage results from nonphysiological loads applied to muscle, muscle can respond by activating a complex response that can lead to successful repair and regeneration of the injured tissue. However, if the magnitude or frequency of muscle damage is too great, injured muscle may not be able to mount a successful program of repair and regeneration; instead, the injured muscle may be replaced by connective tissue. Because of the large number of essential physiological functions served by muscle, insufficient repair or regeneration of muscle can affect the viability of the organism. The broad importance of successful regeneration is emphasized by disease conditions in which muscle regeneration is insufficient, such as muscular dystrophies, that can lead to extensive, systemic functional defects and ultimately death.

The goal of this article is to present current knowledge concerning mechanisms of muscle injury, repair, and regeneration. Although emphasis is placed on injuries that are attributable to mechanical loading, much of our understanding of muscle injury, repair, and regeneration is derived from investigations of muscle's response to injury associated with other pathogenic processes, such as ischemic damage and muscle-wasting diseases; important findings from those other pathogenic processes will also be presented and compared to the response of muscle to mechanical damage.

What is muscle injury?

Skeletal muscle injury is typically defined according to physiological or morphological indices. Most commonly, injury is expressed quantitatively as decrements in force production or disruptions in normal myofibrillar structure. The most rigorous assessments of injury rely on quantitation of the loss of muscle contractile function, such as reductions in force production relative to muscle cross section (specific tension or specific force), increases in time-to-peak force production, loss of peak tetanic force, or increases in fatigability. Morphological indicators of injury can also be quantified, but typically qualitative or semi-quantitative indices have been used, such as notation of apparently increased disruption of myofibrillar structure that is identified by light microscopy. These nonrigorous morphological assessments may underrepresent or overrepresent the presence of injury, especially because disruptions in muscle morphology caused by injury are frequently distributed inhomogeneously throughout the tissue, occurring in focal lesions. Thus, rigorous analysis of muscle injury by morphological assays depends on a well-designed,

stereological assessment of muscle structure by light or electron microscopy.

Although identifying decrements in muscle function by any physiological criterion or by disruptions of structure using morphological criteria is sufficient to show injury, the absence of any particular physiological or morphological defect is insufficient to prove that injury has not occurred. For example, the potential for false, negative conclusions was demonstrated in straight-forward experiments by Street and Ramsey (225) in which single fibers from frog skeletal muscle were injured so that all myofibrils were divided at the injury site, away from which they contracted, leaving a myofibril-free zone in the fiber (retraction zone). Despite the magnitude of the injury, when the portion of the fiber proximal to the injury was stimulated, maximal isometric force was transmitted to the tendon distal to the injury site. Thus, according to one index of injury (the ability to transmit maximum isometric force), no injury occurred. However, according to another index (damage to the myofibrillar structure), massive damage had occurred. Similarly, the maximum tetanic force generated by whole, tibialis anterior (TA) muscles of *mdx* mice, the murine model of Duchenne muscular dystrophy (DMD) was greater than the tetanic force generated by healthy muscle (52), although muscle pathology is rampant by other physiological or morphological criteria. Other investigators have shown that the absolute twitch force and tetanic force produced by *mdx* soleus muscles exceeded those forces generated by healthy muscle, although much of the difference was attributable to larger mass of the *mdx* muscles (94). Assays of whole animal function also reveal the danger of concluding lack of muscle injury when no difference in a single metric of injury is identified. When caged with a running wheel, untrained *mdx* mice did not differ from healthy controls in the distance they ran in a week or in their average daily speed of running (94), even though they had a extensive muscle pathology.

Assessments of sarcolemmal integrity are also used frequently as indices of fiber damage. Matsuda et al. (147) showed that fluorescent, extracellular marker dyes that are normally excluded from the cytosol of healthy muscle fibers *in vivo* can leak into the cytosol of injured fibers, and provide a quantifiable index of the extent of muscle injury. This metric of injury has the advantage of being quantifiable, sensitive, physiologically significant, and applicable to a wide range of muscle injuries and pathologies that include exercise-induced injuries, damage by toxins or freezing, genetic diseases of muscle, and muscle damage caused by ischemia or inflammation. However, these measures of membrane damage to assess fiber injury can also yield misleading data if the data are not quantified, because the amount of marker dye in the cytosol varies over a wide-range from fiber-to-fiber; the value at which the signal threshold is set can tremendously affect the measurement of injury. Furthermore, sufficient sampling according to rigorous stereological parameters is also important for these assays of injury, because lesions in injured muscle are frequently focal.

Acute muscle injuries

Acute muscle injuries are defined as defects in normal muscle structure or function that result from perturbations that are applied over a brief period, such as lacerations, contusions, freezing, burning, or exposure to toxins. Acute injuries can also occur during muscle use and are attributable to the loading conditions that are applied to the muscle that exceed the load-bearing capacity of the muscle. Healthy, vertebrate skeletal muscle can routinely generate stresses that exceed 0.3 MPa at frequencies that exceed 10 Hz without experiencing injury (82, 197). However, stresses within the muscle can be increased several-fold when external loads are applied while a muscle is actively generating force under conditions called lengthening contractions or eccentric contractions. Eccentric contractions are the most common cause of acute muscle injury that is attributable to muscle use.

What mechanical parameters are most important in determining muscle injury during eccentric contractions?

Although exercise-induced muscle injuries most typically occur during eccentric contractions (70, 72, 73 150, 151), the specific mechanical parameter that causes injury under those conditions is disputed. The relative importance of the magnitudes of strain and force to muscle injury were dissected using a clever experimental design in which rabbit TA muscles were subjected to 25% strains applied 900 times in 30 min and then assayed for injury 30 min after the end of the strain cycles, using morphological damage and decrements in force production as indices of injury (135). One group of muscles experiencing this strain protocol was activated simultaneously with the onset of the applied strain, while a second group experienced a delay between the onset of the strain and activation of the muscle. As expected, much higher forces were required to achieve a 25% strain to muscles in the first group, in which the muscles were generating force throughout the strain application. Interestingly, the magnitude of injury did not differ between the two groups despite the large difference in the loads applied, indicating that differences in force application did not produce differences in injury if strain were held constant. However, if the same experimental treatment were applied to muscle using a 12.5% strain instead of a 25% strain, the magnitude of damage was significantly less, and was independent of differences in the magnitude of the applied force. Thus, these experiments showed that strain magnitude was the most important parameter in determining the extent of muscle injury during eccentric contractions, at least under these particular experimental conditions.

Nevertheless, the findings of other investigators indicate that the magnitude of the applied force, rather than the magnitude of the consequent strain is the best predictor of the extent of muscle injury. When 5 stretches were applied to activated rat soleus muscle, using strains that were less than 30% and

allowing 4 min rest between each stretch, peak force was the best predictor of muscle injury, when injury was determined by decrements in force production (262). Strain did not contribute significantly to the variance in force deficit in these experiments.

Other studies have demonstrated that other, derived mechanical variables are the best predictors of muscle injury. For example, application of single strains of variable magnitude to mouse extensor digitorum longus (EDL) muscles that were either nonstimulated or tetanically stimulated showed that work done on the muscle, not the strain or peak force, was the best predictor of muscle injury for both stimulated and nonstimulated muscles (27). In this case, injury was assessed by the presence of disrupted myofibril structure, in addition to decrements of force production caused by the experimental treatment. Subsequent studies indicated that the decrease in force production that occurred in fibers experiencing a single stretch was attributable to damage to the myofibrillar apparatus, and not to disruptions of components of excitation-contraction coupling because the injury occurred in permeabilized muscle fibers in both the presence and absence of calcium (145). Furthermore, the magnitude of force deficit was not affected by strain rate. Application of 5, 10, or 20% strains at strain rates of 0.5, 1.0, or 2.0 lengths/s showed that varying strain rate did not affect damage if the strain magnitude were held constant (143). However, release of creatine kinase from rat soleus muscles experiencing injury caused by eccentric contractions was greater at higher strain rates (265), which may indicate that cell membrane damage is strain rate dependent, while myofibrillar damage is strain rate independent.

Some of the apparent discrepancy between the identification of the mechanical parameters that primarily determine the magnitude of injury in these investigations may be explained by differences in the muscles tested and the treatment protocols. Fast twitch fibers are more easily injured during eccentric contractions than slow-twitch fibers (104, 145), which suggests that there are unknown distinctions in the mechanism of injury between fast and slow fibers. Although rabbit TA (used in reference 135) and mouse EDL (used in reference 27) are nearly entirely fast-twitch fibers, rat soleus (used in reference 262) is exclusively slow-twitch. In addition, the mechanism of injury may differ following multiple cycles of loading (for example, reference 135) compared to injury following a single applied strain (for example, reference 27). Indeed, repetition of loading is a variable that influences the magnitude of injury (96). Despite the variability in the relationships between mechanical loading parameters and the occurrence of muscle injury, important generalizations can be made based on the investigations that are described above. First, muscle injury during eccentric contractions can be caused by mechanical factors, independent of neural, endocrine, or inflammatory factors. Second, mechanically induced injuries are rapid and can result from a single, eccentric contraction. Furthermore, assays using permeabilized fibers indicate that a significant portion of the damage that is reflected in deficits in force production results from direct mechanical disruption of myofibrils.

What are the sites of muscle injury during eccentric contractions?

Muscle injuries during eccentric contractions that lead to complete muscle tears occur primarily at or near myotendinous junctions (MTJs), which are specialized, mechanical junctions at which contractile forces are transmitted from the muscle fiber to the extracellular matrix at the ends of muscle fibers (Fig. 2). However, the specific, microscopic site at which failure occurs at the MTJ is influenced by the activation state of the muscle, and perhaps varies with the muscle experiencing loading or with the species of animal used for assay. While frog skeletal muscles fail along the extracellular surface of

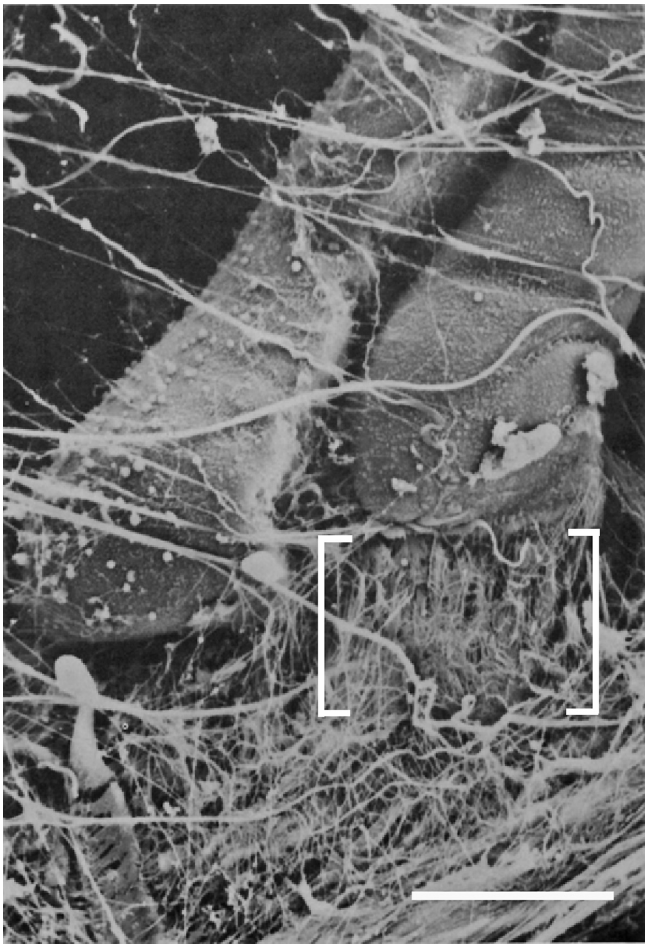

Figure 2 Scanning electron micrograph of two skeletal muscle fibers terminating at their myotendinous junctions (MTJs), where they are mechanically coupled to tendon collagen fibers. Bundles of collagen fibers pass from the tendon in the bottom third of the micrograph to bind to the ends of the muscle fibers at the MTJ (between brackets). During muscle strain injuries, lesions occur at or near the MTJ depending on the state of activation of the fiber and the muscle experiencing the strain injury. Bar = 100 μm. [Reproduced, with permission, from reference (238)].

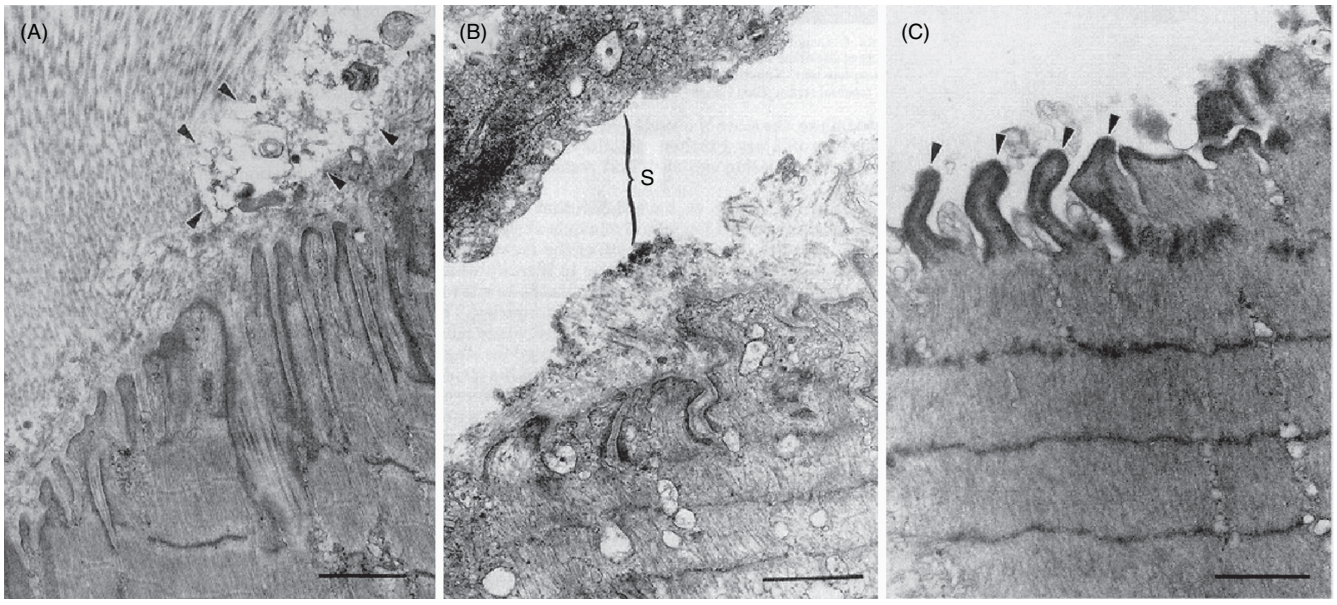

Figure 3 Transmission electron micrographs of longitudinal sections through the myotendinous junction (MTJ) region of frog skeletal muscle that was strained to failure. (A) The upper portion of the micrograph shows tendon collagen fibers. The lower portion of the micrograph shows the MTJ of a muscle fiber that terminates with digit-like extensions of the muscle cell into the tendon. This muscle was unstimulated and shows tissue tears occurred within the connective tissue, near the surface of the muscle fiber (outlined with arrowheads). Bar = 2.0 μm. (B) Complete separations (S) occurred in the connective tissue near the MTJs in unstimulated muscles strained to failure. Other cells in the same preparations show complete lesions within the muscle fiber near the MTJ. Bar = 2.5 μm. (C) Muscles that were strained to failure while stimulated at 20 Hz show that separation in the tissue occurred at the MTJ, along the external surface of the muscle fibers, leaving the digit-like extensions of the cell (arrowheads) protruding into the lesion. Bar = 2.0 μm. [Reproduced, with permission, from reference (242)].

the MTJ during eccentric contractions, failure occurs within the connective tissue near the MTJ in passive muscle that is loaded to failure (Fig. 3) (242). However, rabbit TA muscle that experiences eccentric loading to failure shows separation within the muscle fibers, near the MTJs (Figs. 4 and 5) (76) and the site of loading is not affected by strain rate.

Vertebrate muscles that experience injury during eccentric contractions that do not produce complete muscle tears show large deformations of Z-disks in the myofibrils, called Z-disk streaming, in which the disks display strains of several hundred percent along the longitudinal axis of the myofibrils (Fig. 6) (70, 72, 104, 136, 169, 204). These Z-disk strains can persist for up to 2 weeks following eccentric exercise, suggesting that the damage is corrected by replacement of the Z-disks, and is not reversed by elasticity of the structures. Z-disk deformations during eccentric contractions are presumably caused by loads transmitted through thin and thick filaments, rather than through passive serial elements such as titin filaments or desmin intermediate filaments. Although both titin (261) and desmin (247) form mechanical links between Z-disks in series, Z-disk strain does not occur in nonactivated muscle at the physiological sarcomere lengths at which streaming occurs in eccentric contractions, although streaming will eventually occur at long, supraphysiological sarcomere lengths in passive muscle. During eccentric contractions, when loads are applied to Z-disks via their connections to myofilaments, Z-disks reach their yield point and undergo streaming at relatively low loads, about 22 N/g of muscle (242). In passive muscle, when loads are applied to Z-disks via their connections to titin and desmin, Z-disks do not begin to stream until loads exceed 29 N/g.

Muscle injuries that result from eccentric contractions also involve extensive disruptions to the intermediate filament system that contributes to the passive stiffness of muscle and contributes to maintaining the architecture of myofibrils. Desmin, the most prevalent intermediate filament protein in skeletal muscle, links Z-disks to one another in series, in parallel and to the cell membrane at costameres and at the MTJ (86, 191, 239). Null mutation of desmin increases the compliance of muscles during eccentric contractions (208) and produces defects in coupling between the longitudinal and transverse passive mechanical properties of muscle (22), suggesting a functional role for desmin in integrating the passive mechanical properties of muscle cells. Loss of desmin also reduces muscle fiber stiffness during eccentric contractions (208) and can reduce the susceptibility of muscles to injury during eccentric contractions (208). Eccentric contractions can cause a selective and rapid damage to the desmin intermediate filament lattice that does not occur in strained muscle that is not activated. Immunohistochemical analysis of desmin distribution in cross-sections of rabbit muscle shows that cyclic application of eccentric contractions cause a loss of anti-desmin labeling in a small but significant portion of muscle fibers (71, 137). Some of the loss of desmin from sections of fibers could reflect mechanical damage of the desmin filaments, leading to their retraction away from the desmin-free

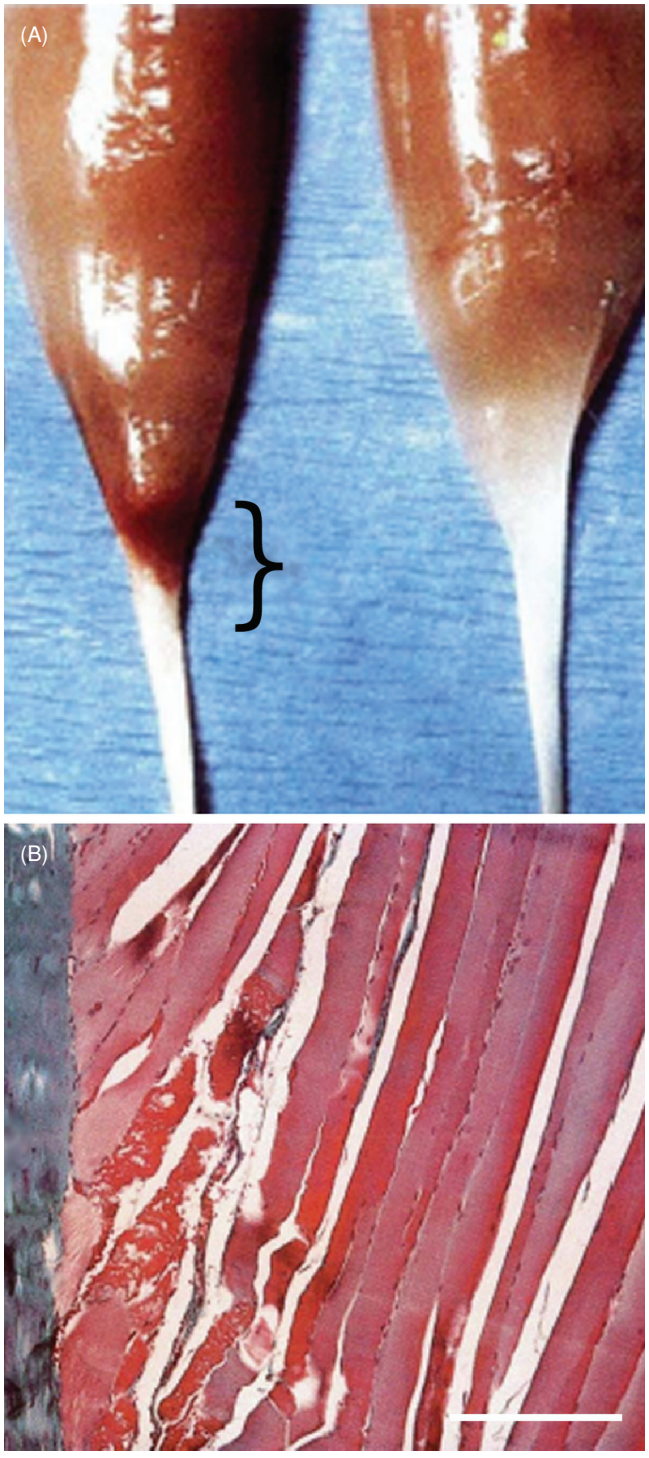

Figure 4 Rabbit tibialis anterior muscle subjected to a strain injury. (A) The muscle on the left was photographed immediately after the strain injury and shows a hematoma (bracket) in the area of the distal, myotendinous junction (MTJ). The muscle on the right is an unstrained control. (B) A longitudinal section of a strained muscle preserved immediately following injury showing the distal MTJ region of muscle fibers (red). The dark, vertical band on the left of the micrograph is tendon. Note that the morphology of the muscle fibers near the tendon is disrupted. Bar = 80 μm. [Reproduced, with permission, from reference (174)].

zones in the injured fibers. However, a more likely explanation may be that the desmin filaments are degraded selectively by proteases. That interpretation is supported by the progressive increase in numbers of fibers containing desmin-free zones, after the completion of eccentric contractions, and by the persistent presence of other myofibrillar proteins in the zones lacking desmin (137). Perhaps the damage to the intermediate filament lattice that occurs preferentially during eccentric contractions reflects a preferential activation of proteases that act on desmin during eccentric loading conditions.

What are the causes and consequences of muscle membrane lesions that occur in injured muscle?

Unregulated calcium-influx through muscle membrane lesions can promote muscle injury by protease activation

Although there are strong data to show that deficits in force production that are caused by injuries during eccentric contraction are primarily attributable to mechanical damage of myofibrils (145), the cell membrane of muscle cells also experiences extensive damage during acute muscle injuries, which can have profound effects on muscle homeostasis. These membrane lesions are large enough to allow the unregulated influx of extracellular marker dyes bound to albumin, a 66,000 Dalton protein, and unregulated efflux of muscle cytosolic proteins such as creatine kinase. However, the increased accumulation of extracellular marker dyes into injured muscle fibers and the increased concentration of cytosolic proteins from muscle in the extracellular space is progressive for days following increased muscle loading or after the application of eccentric contractions (40, 106, 114). That delay indicates that much of the damage to the cell membrane that is caused by muscle loading is caused by factors that are secondary to mechanical damage.

Given the easy transit of large proteins across the damaged membrane, translocation of small ions following their concentrations gradients would also occur rapidly and have major effects on normal regulatory processes within the muscle fiber. In particular, calcium ions that are normally present in the extracellular compartment at about 1.8 mM and within the cytosol at about 0.2 μM, enter injured muscle rapidly through membrane lesions. The magnitude of unregulated influx of cytosolic calcium corresponds to the magnitude of subsequent leakage of cytosolic proteins into the extracellular space (68), suggesting that much of the increase in membrane damage may be secondary to process that are regulated by calcium. Although unregulated influx of this important signaling molecule can lead to immense disruptions of normal homeostasis, activation of calcium-dependent proteases (calpains) within the muscle cytosol appears to be a particularly important pathogenic consequence of membrane damage during muscle injury.

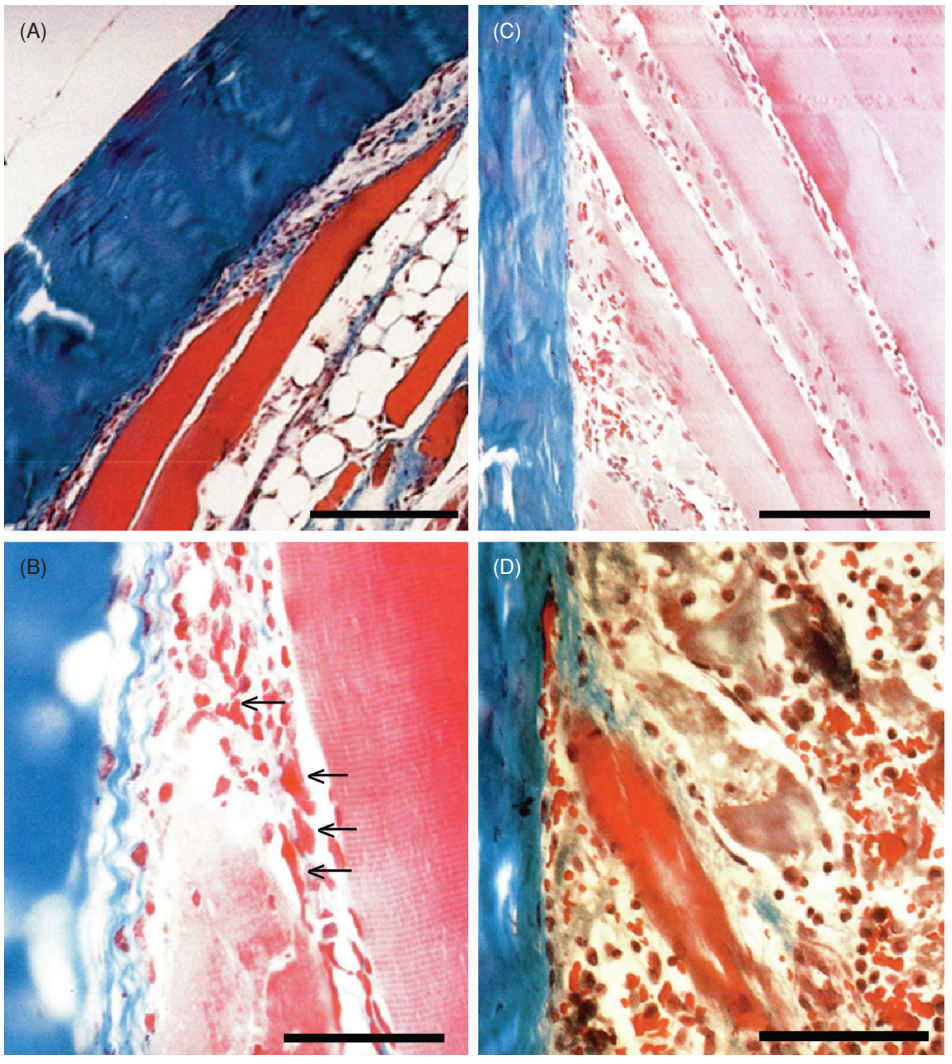

Figure 5 Rabbit tibialis anterior muscle subjected to a strain injury. (A) and (B) myotendinous junction (MTJ) region of strain-injured muscle at 24-h postinjury. Note the separation tissue separation in the MTJ region of the injured muscle between tendon (blue) and muscle fibers (red) (panel A) and the large number of red cells and leukocytes (arrow heads) in the injured MTJ region (panel B). (C) and (D) MTJ region of strain-injured muscle at 48-h postinjury. Increased numbers of leukocytes have entered the sites of tissue separation in the MTJ region (panels C and D) and necrotic fibers (brown) invaded by leukocytes are apparent at the sites of tissue damage (panel D). Bars in upper panels = 100 μm. Bars in lower panels = 50 μm. [Reproduced, with permission, from reference (174).]

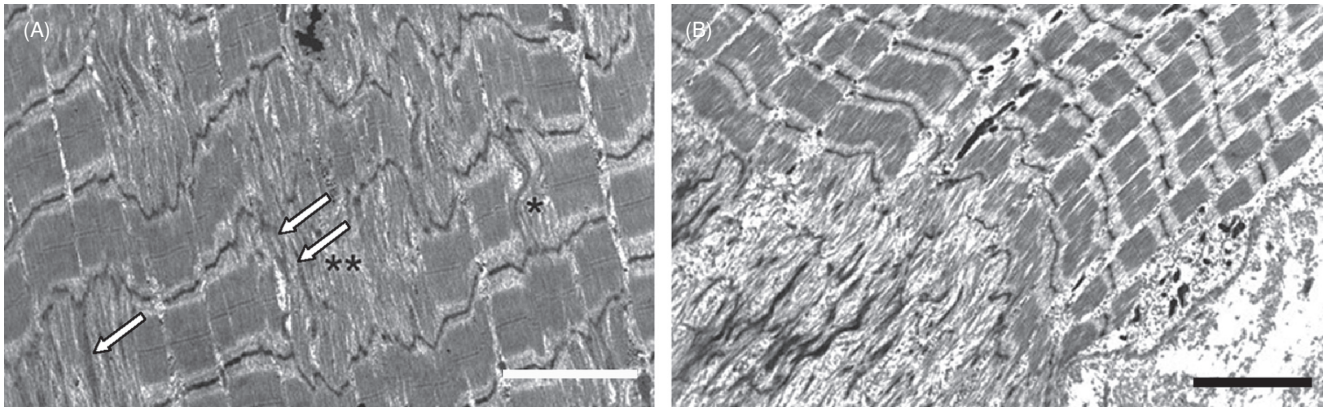

Figure 6 Transmission electron micrograph of human skeletal muscle sampled after injury caused by eccentric contractions. (A) Focal disruption of myofibrillar structure in which Z-disks are no longer aligned, Z-disk streaming is present (arrowhead) and in some cases (*) normal myofibril structure is degraded. Bar = 3.5 μm. (B) Region of an injured muscle fiber that shows extreme degradation of myofibrillar structure, including extensive Z-disk streaming (arrowheads). Bar = 4.5 μm. [Reproduced, with permission, from reference (125)].

Many studies suggest, but do not prove, that calpain activation in injured muscle can promote muscle damage. Elevations in cytosolic calcium concentration would be a key event in the rapid activation of calpain proteolytic activity, and eccentric contractions produce rapid elevations in cytosolic calcium (141, 265) that can persist for more than 1 h after the end of eccentric contractions (10). Calcium influx from the extracellular space provides the primary source of increased cytosolic calcium in stretched muscle (5), although influx of extracellular calcium alone is insufficient for calpain activation. Even in the presence of elevated cytosolic calcium, calpain activity can be negatively regulated by binding of the protease to its endogenous inhibitor, calpastatin (164). Nevertheless, both desmin and α-actinin, the major structural protein in Z-disks, are calpain substrates (167, 200) and the damage to Z-disks and intermediate filaments that occurs in eccentric contractions may partially reflect their proteolysis caused by increased activation of muscle calpains. Collectively, these observations support the conclusion that the unregulated influx of extracellular calcium into injured muscle leads to calpain activation and the subsequent proteolysis of structural proteins that are important for determining the passive mechanical properties of muscle cells and for maintaining myofibrillar architecture. However, deficits in active force production that are caused by eccentric contractions are not likely caused by calpain-mediated cleavage of myofilament proteins, because decrements in force production that are caused by eccentric contractions are not affected by changes in calcium concentration (141).

Unregulated calcium-influx through muscle membrane lesions can promote muscle injury by phospholipase activation

Unregulated calcium influx into injured muscle fibers can also promote muscle damage through mechanisms that are independent of calpain-mediated proteolysis. In particular, activation of phospholipase A2 (PLA_2) by calcium may be especially important for increasing membrane damage in muscle following eccentric contractions or other injuries. PLA_2 is expressed constitutively in muscle and other cells of vertebrates in which it hydrolyzes glycerol at the cell membrane to generate arachidonic acid and lysophospholipids (53). Arachidonic acid can then function as a second messenger or can be metabolized by cyclooxygenase to produce eicosanoids, such as leukotrienes and prostaglandins, that can promote inflammation (53). Thus, PLA_2 can modify membrane structure, modulate cell signaling, and promote inflammation. Normally, PLA_2 function is tightly regulated, and its activation occurs when cytosolic calcium is elevated or when it is phosphorylated by MAP kinase (131).

Several observations indicate that muscle membrane damage is worsened by supraphysiological activation of PLA_2 that is caused by excessive calcium influx into injured muscle fibers (89). For example, the large, rapid efflux of cytosolic proteins such as creatine kinase that occurs following treatment of muscle with a calcium ionophore is effectively blocked by inhibiting phospholipase activity with chlorpromazine or inhibiting lipoxygenases with NDGA (60) and the application of purified PLA_2 to skeletal muscle causes muscle membrane lysis and microscopically discernible holes in muscle cell membranes (89). Furthermore, increases in PLA_2 activation can cause damage to the structure of myofibrils that resembles defects caused by damaging eccentric contractions. Application of PLA_2 to muscle produces a rapid loss of desmin from regions of injured fibers (88, 92) and disruption of Z-disks (81). Although PLA_2-induced membrane damage is likely a direct effect of PLA_2 interactions with the cell membrane, the cytoskeletal defects are likely not the direct consequence of PLA_2-mediated events. Neither chlorpromazine nor lipoxygenases prevented signs of myofibrillar injury, such as Z-disk streaming, in calcium ionophore-treated muscles (59, 60) or in demembranated muscle fibers placed in high calcium solutions (59).

Can phospholipase activation promote muscle membrane injury through free-radical-mediated processes?

The production of free radicals in skeletal muscle is modulated by the contractile state of muscle, which provides a mechanism through which signaling pathways can be regulated as a function of muscle activity. For example, production of superoxide and nitric oxide (NO) is low in resting muscle, but increases rapidly to levels 10-fold greater soon after the onset of contraction (9, 168, 184, 201, 241). The induction of free radical production in contracting muscle, particularly the increased synthesis of superoxide and its metabolites, can be nearly completely ablated by inhibitors of PLA_2 (168), which shows a link between PLA_2-mediated signaling and free radical production that may be functionally important. Furthermore, selective inhibition of calcium-independent PLA_2 did not reduce free radical production (168), indicating that the calcium-dependent form of PLA_2 was most important in this role, and that modulation of cytosolic calcium can influence free radical production through a PLA_2-mediated pathway.

Whether supraphysiological activation of PLA_2 in injured muscle can elevate free radical production to levels that increase muscle damage has not been tested explicitly. However, the muscle cell membrane provides an especially vulnerable target for free-radical-mediated damage because lipids, and polyunsaturated fats in particular, are highly reactive with free radicals (33, 149). The potential cytotoxicity of superoxide and its metabolites suggests that PLA_2 activation could feasibly contribute to muscle damage through this process. In addition, administration of superoxide dismutase to rats that were subjected to muscle strain injury decreased creatine kinase in the serum 3-day postinjury (132), although this treatment would not selectively deplete superoxide that was derived from PLA_2-mediated, intracellular processes or produced by muscle cells rather than other types, such as inflammatory cells. Nevertheless, some experimental findings cast doubt on

a potential role for superoxide production that is driven by PLA_2 activation in contributing to muscle membrane injury. Muscle injuries caused by eccentric contractions of mouse EDL muscles, as assessed by force deficits caused by injury, did not differ significantly between wild-type mice and mice that over-expressed superoxide dismutase or catalase within the muscle cytoplasm (193). These findings suggest that any free radical-mediated damage to the injured fibers is not attributable to free radicals that are generated within the muscle fibers, at least in this injury model when assessed by these indices of damage.

Inflammatory cells play a key role in promoting muscle membrane damage via free radical-mediated mechanisms

The delay in the appearance of cytosolic proteins from muscle in the serum of animals following increased muscle use or muscle injury indicates that much of the membrane damage is not a direct consequence of mechanical stresses, but instead, membrane damage is largely attributable to some other injury mechanism. The mechanism of muscle membrane damage during modified muscle use *in vivo* has been explored in a model of modified muscle use in rodents in which hindlimbs experience a removal of weight bearing for a period of time, typically 10 to 14 days, and then are returned to normal weight bearing (the unloading/reloading model). Hindlimb muscles, especially the soleus, lose muscle mass during unloading and the subsequent return to reloading causes mild muscle injury that occurs in normal locomotion in unanesthetized animals *in vivo*. However, most membrane lesions do not occur at the onset of reloading; instead, membrane lesions increase over a period of several days following the onset of reloading (106).

Several observations supported early expectations that a significant proportion of muscle fiber damage, especially muscle membrane damage, may be attributable to the cytolytic activities of inflammatory cells. Following eccentric exercise in humans, the first morphologically discernible signs of muscle damage postexercise coincided with the invasion of neutrophils into the muscle, and the distributions of neutrophils and injured fibers were similar (65). Similarly, the rodent unloading/reloading model showed coinciding distributions and time of occurrence of neutrophil invasion and fiber damage in soleus muscles (240), again suggesting that neutrophils may promote muscle membrane damage, and not simply remove cellular debris produced by muscle injury. Furthermore, administration of an antibody that blocks the respiratory burst and degranulation of neutrophils prior to eccentric contractions caused great reductions in morphologically discernible damage to muscle (26). Nevertheless, a previous investigation showed that there was no difference in muscle injury caused by eccentric contraction of the muscles of neutrophil-depleted and normal mice (142). However, this discrepancy may be attributable to differences in the treatments used to produce injury. Lowe et al. (142) used 150 lengthening contractions which would have produced extensive mechanical damage that could overwhelm a neutrophil-mediated effect, while whereas Brickson et al. (26) used a single eccentric contraction.

The finding that administration of superoxide dismutase to experimental animals reduces muscle membrane damage that occurs following strain injuries (132) and the observation that overexpression of cytosolic superoxide dismutases in muscle fibers does not protect against muscle fiber injuries caused by eccentric contractions (193) suggest that superoxide produced outside of the muscle fiber, perhaps by neutrophils, contributes to muscle membrane damage during injury. Neutrophils are rich sources of superoxide, primarily generated by NADPH oxidase that is activated as part of the respiratory burst in neutrophils. Neutrophil lysis of muscle cells *in vitro* is greatly reduced by the presence of exogenous superoxide dismutase (171), or by null mutation of the catalytic subunit of NADPH oxidase ($gp91^{phox}$) (172), providing evidence that neutrophil-derived superoxide metabolite can play a significant role in muscle membrane damage. Free radical production by neutrophil NADPH oxidase also contributes importantly to muscle membrane lysis *in vivo*. Ablation of the $gp91^{phox}$ gene in mice that were subjected to hindlimb unloading/reloading nearly eliminated muscle membrane lysis during the reloading period (172), showing that superoxide or one of its metabolites is a major source of muscle membrane lysis during modified muscle use. However, much of the membrane lysis was probably not directly attributable to superoxide, but instead resulted from cytolysis by hypochlorous acid. Null mutation of myeloperoxidase (MPO), which catalyzes the formation of hypocholorous acid from superoxide-derived hydrogen peroxide, prevented most of the muscle membrane lysis that occurred in reloaded soleus muscles *in vivo* (170). Surprisingly, exercise may promote MPO-mediated cytotoxicity by neutrophils. A positive relationship between exercise and MPO in sera has been demonstrated (15, 29), and at least some of this change was attributable to an increase in MPO release per neutrophil during exercise *in vivo* (231). Furthermore, application of mechanical loads to co-cultures of muscle cells and neutrophils increased the release of MPO and increased muscle cell lysis through MPO-mediated mechanisms (170).

Disruptions of vascular perfusion can play a key role in promoting muscle membrane damage via free radical-mediated mechanisms

Loss of normal vascular perfusion of skeletal muscle, even for relatively brief periods, can produce serious muscle damage, much of which is caused by cytotoxic and cytolytic actions of free radicals. However, loss of perfusion *per se* is not the direct cause of most muscle membrane damage that results from temporary loss of blood flow to muscle; reperfusion of the muscle following periods of temporary ischemia is the primary cause of injury, and most of the damage is largely attributable to the release of free radicals from inflammatory cells (115, 120, 187, 188, 274). Thus, skeletal muscle injuries

caused by periods of ischemia followed by reperfusion (IR) provide a valuable model for exploring relationships between inflammation, oxidative stress, and muscle damage that are not caused by mechanical perturbations applied to muscle.

Several investigations have provided evidence that free radicals generated by neutrophils play a major role in damage to the muscle cell membrane during IR. Early studies showed that the onset and severity of muscle damage during IR corresponded with the kinetics of neutrophil invasion. Furthermore, systemic depletion of neutrophils prior to IR caused large reductions in histologically discernible muscle damage and reductions in the release of cytosolic proteins from muscle cells into the circulation, which reflected the extent of muscle membrane damage (115, 120). Muscle damage during IR was also reduced by blocking the function of adhesion molecules that mediate neutrophils adhesion to the vascular endothelium, which is a prerequisite for their extravasation (diapedesis). Systemic administration of antibodies to integrins, especially CD18, or to selectins that are involved in neutrophil-adhesion to the vascular endothelium reduced neutrophil diapedesis and also significantly decreased muscle injury caused by IR (144, 188, 215, 273). However, leukocyte functions that are mediated by CD18 or selectins are not limited to neutrophils. Other leukocytes that include monocytes, macrophages, mast cells, and basophils express these ligands and demonstrate impaired functions when CD18 or selectin binding is blocked or CD18 or selectin expression is disrupted (57, 85, 154, 222, 236, 250).

Superoxide or a superoxide-derivative generated by neutrophils likely contributes to muscle membrane damage that occurs during IR. Neutrophils show enhanced production of superoxide during reperfusion (67) and defects in muscle membrane resting potential that were caused by IR were prevented by systemic treatments with superoxide dismutase and catalase (284). Furthermore, administration of a polyoxyethelene-modified superoxide dismutase to experimental animals prior to reperfusion of ischemic muscle prevented the increase in muscle of lipid peroxides (107), an indicator of oxidative damage to cell membranes. While the identity of the specific free radicals that were directly responsible for membrane damage during IR is uncertain, assays of free radicals present in the muscle interstitium during IR showed significantly elevated hydroxyl radicals, with no increase in superoxide (183). This finding suggests that hydroxyl radicals derived from superoxide are more likely to be the damaging reactant, although disruptions in superoxide production or metabolism could have downstream effects on the level of hydroxyl radicals in muscle.

Other leukocytes may contribute importantly to muscle membrane damage during IR. Mast cells are also elevated at early stages of reperfusion and they are likely to promote muscle damage during IR by the release of histamine into the reperfused muscle, contributing to edema or by production of cytotoxic free radicals. Data obtained from genetically modified mice support the likelihood that mast cells induce muscle damage during IR. The mast cell-deficient line that was used in these investigations (W^f/W^f mice) arose from a spontaneous mutation in the gene encoding c-kit receptor tyrosine kinase (32, 77). Expression of c-kit in fully differentiated cells occurs only in mast cells and eosinophils, and loss of c-kit expression yields a reduction of mast cell numbers by more than 50% (252). Mast cell-deficient mice that were subjected to IR had higher levels of lactate dehydrogenase (LDH) in reperfused muscle than occurred in the muscles of wild-type mice subjected to IR (127). Because LDH is a cytosolic protein in muscle fibers that leaks out of muscle cells in which the cell membrane has been lysed, retention of higher levels of LDH in muscle indicated less membrane damage. Furthermore, mast cell deficiency did not affect the numbers of neutrophils in the reperfused muscle or the concentration of MPO (126), which showed that the protective effect of the mutation was not attributable to a reduction in neutrophil numbers. Subsequent findings confirmed that the protective effect of the mutation in mast cell-deficient mice was specifically attributable to mutation of the targeted gene in cells in the hematopoeitic lineage. Transplantation of wild-type bone marrow cells into mast cell-deficient mice prevented the protective effect of mast cell deficiency on IR injury (23). However, a portion of the protective effect of the mutation in mast cell deficiency may be attributable to the effect of the mutation on nonmast cells. C-kit is also expressed on common myeloid progenitors, common lymphoid precursors and myeloblasts, which are progenitors of basophils, neutrophils, monocytes and macrophages. Thus, it is feasible that disrupted expression in c-kit in myeloid progenitors could affect the functional capacity of myeloid cells other than mast cells that have not yet been identified.

Mast cell-derived NO is expected to make large contributions to muscle injury caused by mast cells during IR. Observations that support this role for mast cells include the finding that mast cells that are present in muscle during IR express high levels of inducible nitric oxide synthase (iNOS) (155), NO produced by myeloid cells can lyse muscle cell membranes (171) and ablation of iNOS expression in mice subjected to IR reduces muscle damage (14). However, other myeloid cells that are elevated in muscle during IR, such as neutrophils and macrophages, also express iNOS and could contribute to iNOS-derived cytolysis. Furthermore, NO plays a complex role in the response of muscle to IR, and there is strong evidence that NO can both contribute to damage, as well as protect against damage in IR. On one hand, NO can cause cell membrane damage, either through direct actions as a free radical, or through its subsequent conversion to another free radical, such as peroxynitrite ($ONOO^-$); this would be expected to worsen muscle cell damage during IR. On the other hand, NO can reduce the expression of adhesion molecules that mediate the binding of leukocytes to the vascular endothelium, which is required for leukocytes to extravasate into muscle during IR; this effect would be expected to be protective against myeloid cell-induced damage to muscle. Experimental evidence for the protective effect of NO against inflammation of muscle during IR was demonstrated

in rats that received systemic administration of a NOS inhibitor and L-arginine, the substrate for NOS that can increase NO production (75). L-arginine treatment prior to reperfusion reduced leukocyte adherence to the endothelium but the treatment effect was prevented by the co-administration of NOS inhibitor. Thus, inhibition of leukocyte diapedesis by elevating NOS activity with supplemental arginine would be expected to reduce myeloid cell damage to muscle during IR, which has been reported by other investigators (100, 153). However, others reported that NOS inhibition also reduced IR injury to muscle (112). These apparently conflicting results may be attributable to the differing functions of NO molecules derived from different NOS isoforms generated at distinct cellular or subcellular sites during IR. Perhaps the protective effect of NOS inhibition reflected the loss of the cytolytic activities of iNOS-derived NO on myeloid cells, while the protective effect of arginine supplementation could reflect the anti-adhesive effects of endothelial NOS (eNOS)-derived NO on endothelial cells. This latter speculation is supported by the finding that over-expression of eNOS reduces neutrophil numbers in reperfused muscle, and increases muscle viability following IR (180).

Muscle membrane damage caused by the activation of complement

The innate immune system is able to induce muscle membrane lysis and muscle fiber injury through activation of the complement system (Fig. 7). The complement system consists of a large family of soluble proteins in the serum that are normally present in a monomeric, inactive state. However, infection or trauma can lead to activation of proteases that cleave complement proteins into fragments that are biologically active. Although there are three separate pathways that can each lead to activation of the system, each pathway can yield formation of a membrane-attack complex that can cause cell lysis and death when the complex is assembled in a target cell membrane (162, 210). Other specific proteolytic fragments that are produced by complement activation can play important, regulatory roles for the function of the innate immune system. For example, C3a and C5a can enhance expression of adhesion molecules that enable neutrophil adhesion to endothelial cells in advance of diapedesis. C3a and C5a are also chemoattractants for neutrophils and macrophages (105) and they stimulate the release of histamine by mast cells (156). C5a also increases the production of free radicals by neutrophils (36, 79) and promotes the secretion of proinflammatory cytokines by neutrophils and macrophages (30, 61, 80). Thus, complement activation could increase muscle membrane damage through direct lysis or by promoting the numbers or activities of myeloid cells that can increase damage through free radical-mediated mechanisms.

Complement activation occurs in several neuromuscular diseases and in muscle injury. For example, complement activation occurs in muscular dystrophy (220), dermatomyositis (110) and myasthenia gravis (251) and complement cleavage products C3a and C4a are present at elevated levels in the in sera of subjects after prolonged exercise (58). In at least some cases, activated complement in skeletal muscle contributes significantly to muscle injury and muscle membrane lysis. During the rodent hindlimb unloading/reloading model, the complement system is activated early in the reloading process (69). However, blocking complement activation by administration of a soluble form of complement receptor-1 (sCR1), a ligand of C3b, was sufficient to reduce the numbers of neutrophils and macrophages in the reloaded muscle and also produced significant reductions in muscle fiber necrosis (69). Although these findings indicate a role for complement in muscle fiber damage during modified muscle use, whether the complement-mediated damage occurred primarily through direct lysis of muscle cell membranes via formation of the membrane-attack complex or by attracting cytolytic myeloid cells into the muscle was not tested.

Activation of the complement system also contributes to muscle injury during IR. Treating mice with sCR1 prior to the reperfusion of ischemic muscle or the genetic ablation of C5 expression in mice that were subjected to IR caused reductions in muscle injury by nearly 59% (120). Much of this complement-mediated damage occurred through the classical pathway of complement activation that is strongly activated by immunoglobin M (IgM) bound to antigen which in turn binds complement receptor-2 (CR2) on the surface of B cells. Mice that were null mutants for CR2 were largely protected from histologically discernible injury in IR (7). Because previous investigations had shown that CR2 mutations protected other tissues from IR injury but that the protective effect could be reversed by systemic administration of B-1 lymphocyte-derived IgM (66, 202), whether the CR2-mediated muscle damage that occurred in IR similarly relied on B-1 lymphocyte-derived IgM was tested. As expected, replenishment of B-1-derived IgM reversed the protective effect of the CR2 mutation in skeletal muscle IR injury (7), demonstrating a significant role of classical activation of the complement system in IR injury of skeletal muscle. This complement-mediated damage is additive to neutrophil-mediated damage in IR. Blocking the complement cascade through C5-deficiency and sCR1 treatments reduced muscle damage by nearly 50% in IR and neutrophil-depletion in wild-type mice produced a reduction in injury of about 38% in IR, but combining neutropenia and C5-null mutation reduced muscle injury more than 70% (120), showing that muscle injury by neutrophils or the complement system occurs through two, largely independent processes in IR.

Mechanisms of muscle repair and regeneration

Muscle repair and regeneration that follow injury or disease requires that muscle first re-establish homeostasis following the damaging perturbation and then restore the normal structural and functional capacities of the fully differentiated tissue. As the preceding sections on muscle injury emphasized, breakdown of the integrity of the muscle cell membrane is a

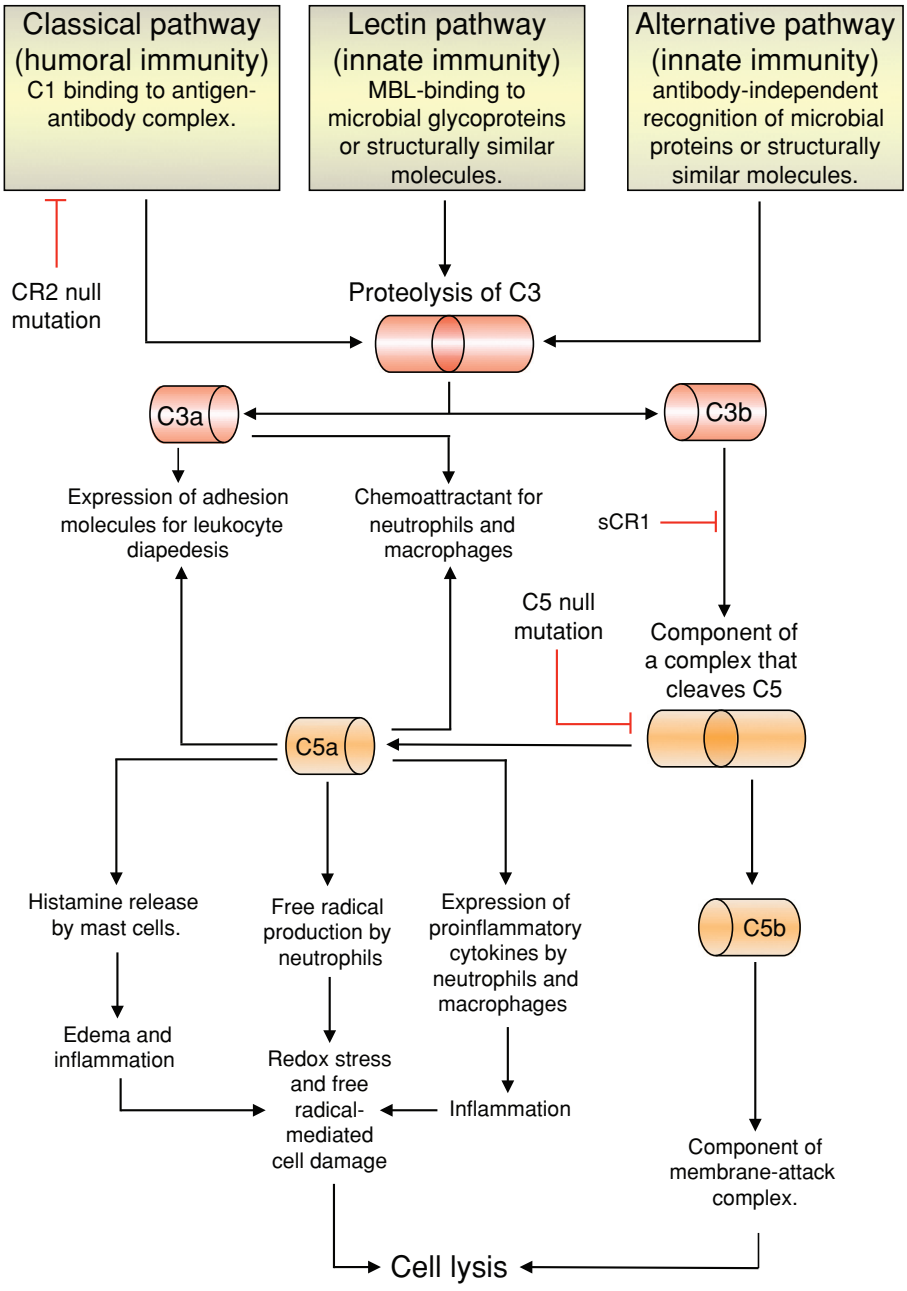

Figure 7 Simplified schematic representing some of the steps of the complement cascade that have been implicated in skeletal muscle injury. Current experimental data support a role for the classical pathway, a component of humoral immunity, and the alternative pathway, a component of innate immunity, in causing muscle damage. The potential involvement of the lectin pathway has not been explored. Each pathway progresses through multiple steps to lead to the proteolysis of C3. The products of C3 cleavage, C3a and C3b, can contribute to muscle inflammation and injury through multiple effects. C3a released into the extracellular space can increase the expression of adhesion molecules on the surface of leukocytes and endothelial cells that enables the more rapid extravasation of leukocytes into injured tissue. C3a can also act as a chemoattractant to bring more myeloid cells to the site of injury. C3b can amplify muscle inflammation and damage by participating in the cleavage of C5. C5a that is generated by C5 cleavage can increase histamine release by mast cells, which drives edema and further inflammation that can promote muscle damage. C5a can also increase the production of potentially cytotoxic free radicals by neutrophils and elevate the expression of Th1 cytokines, which could further elevate inflammation and inflammatory cell-mediated damage to muscle cells. C5b contributes to muscle cell damage by associating with other proteins in the complement system to form a transmembrane, membrane-attack complex that allows the unregulated flux of ions and small molecules across the target cell membrane, leading to cell lysis. Experimental data show that muscle cell damage during IR is less in CR2 null mice, which would have impaired activation of the classical pathway (7). Treatment of mice subjected to IR with sCR1 to block C3b or genetic ablation of C5 (120) also reduced muscle damage, confirming involvement of the complement system in the injury, although not discriminating between specific pathways. Similarly, treatments with sCR1 reduce muscle damage in unloading/reloading (69).

common feature of muscle injury and disease that produces tremendous disruptions in normal homeostasis. Thus, restoration of membrane structure and function is a key, early feature in successful repair and regeneration of muscle. However, muscle membrane damage itself can initiate signaling processes that activate muscle regeneration. The synthesis and release of signaling molecules that are activated by muscle fibers subjected to membrane damage can induce the activation and attraction of a diverse population of cells that promote muscle regeneration and growth. Following acute muscle injury, the sequence of muscle injury-repair-regeneration leads to full functional recovery with days to weeks of the initial damage. However, if injuries are chronic, successful regeneration may never be achieved.

Repair of the muscle cell membrane

Despite the necessity of successful repair of damaged muscle cell membranes for the re-establishment of muscle homeostasis following injury, current understanding of the mechanisms of repair is rudimentary. However, advances in this field have been stimulated by the discovery that some inherited, progressive muscular dystrophies that include limb girdle muscular dystrophy 2B (LGMD2B) and Miyoshi myopathy can be caused by null mutation of a gene that encodes a protein involved in membrane repair. The deficient gene product, a protein called dysferlin (138), is located in the cytoplasm and at the cell surface of muscle fibers and dysferlin-null mice show an increased occurrence of muscle fibers that contain elevated concentrations of tracer molecules normally restricted to the extracellular space (11). However, exercise does not increase membrane damage in dysferlin-null mice (11), which suggests that the increased membrane damage in dysferlin-deficient mice does not reflect increased susceptibility of the membranes to mechanical damage. Instead, loss of dysferlin slows the repair of damaged membranes. For example, dysferlin-null fibers that were damaged by irradiation in isolated fiber preparations resealed their damaged sarcolemma more slowly than wild-type fibers injured by the same process (11). Similarly, muscles of dysferlin-mutant mice that were damaged by forced-strain injuries were slower in their ability to repair the muscle membrane's ability to exclude extracellular-tracer dye entry into the cytoplasm (206).

Morphological observations indicate a role for dysferlin in membrane sealing by mediating the fusion of cytosolic vesicles with the surface membrane of muscle cells. For example, immunohistochemistry shows cytosolic, punctate staining of muscle fibers of wild-type mice labeled with anti-dysferlin, in addition to staining of the fiber surface (11), suggesting that dysferlin is present on vesicles in addition to being located at the cell surface. Electron microscopy of dysferlin-deficient muscles shows an accumulation of vesicles deep to the plasma membrane of muscle fibers and an increased frequency of gaps in the sarcolemma (31, 216), which has been interpreted as reflecting an impaired fusion of the vesicles with the surface membrane, leading to defects in plasma membrane repair.

Membrane-resealing that is mediated by dysferlin is positively regulated by cytosolic calcium, which may be relevant in the context of membrane repair following fiber injury, when cytosolic calcium would increase because of its unregulated influx through membrane lesions. Isolated fibers from wild-type or dysferlin-deficient mice showed similar kinetics for membrane repair following radiation-induced damage when repair occurred in the absence of calcium (11). However, repair of membrane damage was significantly more rapid in the presence of 1 mM calcium in wild-type fibers than in mutant fibers. The calcium dependency of dysferlin's function may be manifest in several of dysferlin's interactions. Dysferlin binding to membrane phospholipids themselves shows a calcium dependency (49) and dysferlin's interactions with other membrane proteins is also positively regulated by calcium. For example, dysferlin-binding to the membrane-associated proteins annexin A1 and annexin A2 is calcium-dependent (130), and annexins also bind phospholipids and function in membrane trafficking (199), which could contribute to membrane resealing.

Although the growing knowledge of dysferlin's role in muscle membrane repair is providing important new insights into mechanisms that underlie the ability of muscle fibers to recover normal membrane integrity and homeostasis following injury, findings also show that dysferlin is unessential for muscle membrane repair. The functional defects of LGMD 2B and Miyoshi myopathy do not become apparent until mid-childhood or later and only some muscle groups are involved (78). Similarly, dysferlin-deficient mice show a mild phenotype (11) and do not display exercise intolerance (37), which would be expected if dysferlin were essential for repair of membrane damage caused by exercise. Together, these findings show that dysferlin-independent mechanisms for muscle membrane resealing exist and that their relative importance in membrane repair varies between muscles and with age.

Satellite cell contributions to muscle repair and regeneration

Muscle injuries frequently cause the activation of a quiescent population of myogenic cells, called satellite cells, that reside in the space between the muscle cell membrane and the basal lamina that ensheathes each individual muscle fiber (41, 148). Muscle regeneration that involves satellite cell activation proceeds through three general stages in which satellite cells are first activated and proliferate, then withdraw from the cell cycle to begin differentiation and finally proceed to form fully differentiated muscle fibers (Fig. 8). Each stage of regeneration is regulated in part by proteins in a family of muscle-specific transcription factors, the basic helix-loop-helix family (bHLH), that includes MyoD, myogenin, Myf4, and Myf5 (reviewed by references 128, 139). Heterodimerization of these bHLH proteins with specific enhancer proteins enables their binding to muscle-specific genes to activate their expression.

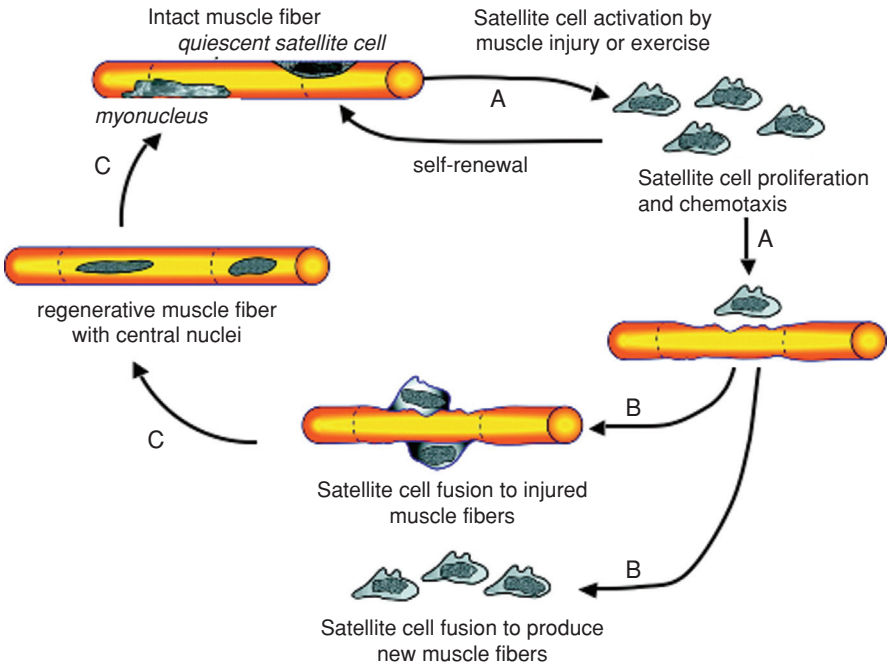

Figure 8 Schematic representation of the cycle of satellite cell activation, proliferation, and differentiation following muscle injury. Acute trauma of muscle fibers induces activation of satellite cells that normally reside in a quiescent state between the exterior surface of the muscle fiber's plasma membrane and the enveloping basal lamina. Activated satellite cells can then enter the interstitium between muscle fibers and proliferate to expand their numbers. Arrows marked "A" represent events that occur during the activation/proliferation stage of regeneration. Some of the activated cells then exit from the cell cycle and return to their niche as quiescent satellite cells to renew and maintain the satellite cell population. Other satellite cells migrate to the site where muscle repair and regeneration proceed, where they withdraw from the cell cycle to undergo differentiation. Arrows marked "B" represent events that occur during the early differentiation stage of regeneration. As part of their program of differentiation, they can fuse with existing fibers to contribute new nuclei to the fibers or fuse with other mononucleated myogenic cells to form new fibers. During these later stages of muscle regeneration, muscle nuclei align along the longitudinal axis of the muscle fiber, becoming central nuclei which is a characteristic of myotubes or muscle fibers experiencing repair after injury. Arrows marked "C" represent events that occur during the terminal differentiation stage of regeneration. Upon completion of terminal differentiation, the central nuclei migrate to the surface of the muscle fiber. [Adapted and modified, with permission, from reference (93)].

The first stage of muscle regeneration, called the proliferative stage, consists of satellite cell activation and proliferation during which the expression of MyoD and Myf5 is elevated (43, 44, 74, 87, 280). However, expression of MyoD is not a specific indicator of satellite cells in the proliferative stage of regeneration because MyoD can be expressed in quiescent satellite cells, in which MyoD is bound to inhibitory proteins. Similarly, Myf5 is expressed by inactive satellite cells at the proliferative stage of regeneration. As satellite cells exit the cell cycle and enter the second stage of regeneration, called the early differentiation stage, MyoD and Myf5 are activated and increase the expression of muscle-specific genes. Myogenin and Myf4 expression is also initiated during the early differentiation stage, along with transcription factors in the myocyte enhancer binding factor-2 (MEF2) family (43, 44, 74, 87, 158, 280). The expression of gene products that is driven by these transcription factors is necessary for the fusion of myogenic cells to form multi-nucleated myotubes and for the transition to terminal differentiation, the third stage of regeneration. Muscle-specific genes continue to be expressed at high levels during terminal differentiation as myotubes grow and differentiate into fully mature muscle fibers.

Satellite cell activation following muscle injury

A complex mixture of factors link muscle injury with the activation, proliferation, migration, and differentiation of satellite cells that lead to successful repair and regeneration. The earliest studies of satellite cell activation in response to damage showed that injury caused the release of mitogens from the muscles themselves. Crushed muscle extracts applied to muscle fibers that were isolated with their accompanying satellite cells showed that substances released by the damaged muscle rapidly stimulated mitosis of satellite cells (17). Subsequent work showed that transforming growth factor-β (TGFβ), fibroblast growth factor (FGF), and insulin-like growth factor-1 (IGF-1), all of which can be synthesized by muscle, were able to influence both the proliferation and differentiation of

satellite cells *in vitro* (2, 3), suggesting that these previously identified growth factors could also act as mitogens for satellite cells in injured muscle. However, the speed with which satellite cells respond to injury or exercise to re-enter the cell cycle is too rapid to rely on *de novo* synthesis of proteins. Thus, if any of the growth factors that were shown to influence satellite cell activation *in vitro* were to serve those functions immediately following muscle injury *in vivo*, the factors would need to be present in uninjured muscle in an inactive form or sequestered from their cognate receptors. Release of those factors upon muscle injury could enable them to function immediately as "wound hormones" to activate satellite cells and initiate muscle repair and regeneration.

Subsequent studies showed that FGF2 could function as a wound hormone in injured muscle *in vivo*. Downhill treadmill running that would produce eccentric loading on selected limb muscles produced a reduction in FGF2 that was present within the cytoplasm of muscle fibers and that was detectible by immunohistochemistry (39). The treadmill running conditions also caused increased influx of extracellular tracer dye, suggesting that the loss of cytosolic FGF2 resulted from leakage through exercise-induced injuries to the cell membrane. Subsequent work showed that FGF2 released from mechanically loaded muscle cells could promote myogenic cell proliferation. Muscle cells that were subjected to stretching *in vitro* showed a loss of FGF2 into the media, that was associated with an increase in the mitogenic activity of stretched-cell conditioned media (38). However, the mitogenic activity could be significantly reduced if the media were treated with neutralizing antibodies to FGF2, providing evidence for a functional link between the mechanically induced release of FGF2 and muscle cell proliferation (38).

Inactive wound hormones may also reside in the basal lamina and endomysium that surrounds muscle fibers, awaiting activation by exercise or injury. In particular, FGF2 binds to heparin sulfate proteoglycans (HSPs) in the connective tissue that surrounds muscle fibers (55), and the release of select metalloproteinases (MMPs), especially MMP 2, 3, and 9, can cleave HSP core protein, leading to the release of FGF2. Skeletal muscle expresses and can store or secrete MMP 2, 3, and 9 (109, 254) so that any of these MMPs could readily cleave HSP and release FGF2 following muscle injury, leading to satellite cell activation.

Immediate activation of satellite cells following muscle injury or exercise could also result from increased production of NO by NOS. Skeletal muscle expresses neuronal NOS (nNOS), much of which is located at the sarcolemma (113, 165) where the enzyme is activated by calcium and calmodulin (24). Calcium that enters skeletal muscle from the extracellular space is primarily responsible for nNOS activation that is caused by increased muscle activation or by passive stretches applied to excised muscle preparations (241). Recent evidence indicates that muscle-derived NO may contribute to rapid activation of satellite cells by increasing the activation and expression of MMPs. For example, treating satellite cells with an NO-donor-induced cleavage of the inactive proenzyme form of MMP2 to its active form, and the activation of MMP2 that was caused by mechanical stretching was reduced by NOS inhibition (282).

Although a portion of satellite cell activation that occurs downstream of MMP activation may reflect the release of FGF2, a series of interesting investigations has shown that MMP-catalyzed release of hepatocyte growth factor (HGF) from the cell surface is an important mechanism leading to satellite cell activation. HGF is expressed by mesenchymal cells (224) and can be stored in the extracellular matrix, including the basement membrane, by binding to HSPs (166). Thus, activation of MMPs such as MMP2 could cause HGF release as a consequence of HSP core protein cleavage. HGF can then bind its receptor, c-met, which is expressed by satellite cells and triggers activation and proliferation of satellite cells following its ligation (44, 233).

Several experimental observations support a mechanism for satellite cell activation that rapidly follows muscle exercise or injury in which increased muscle NO production activates MMP-2 to yield HGF release, binding to c-met and activation of satellite cells (Fig. 9). First, stimulation or stretching of skeletal muscle cells increases NO synthesis and release (241) and promotes satellite cell proliferation (219, 253), suggesting a possible link between NO release and satellite cell activation. Furthermore, inhibition of nNOS diminished the mitogenic effect of stretch (219). As noted above, MMP activity can contribute to the release and activation of HGF and NO can promote the activation of MMP2 (282). In addition, cell stretching *in vitro* also increases HGF release into the culture media and NOS inhibition reduces HGF release and diminishes satellite cell activation. Treatment of unstimulated satellite cell preparations with conditioned media from stretched cell cultures increases proliferation of the unstimulated cells, and treating stimulated cells with neutralizing antibodies to HGF prevents stretch activation of the cells (234, 235).

Myogenic cell migrations to sites of repair and regeneration

Severe muscle injuries that are caused by crush, toxin exposure, burns, or freezing can deplete satellite cells at the injury site, in addition to causing necrosis of muscle fibers at the site. Regeneration of muscle at sites of severe muscle injury can rely, in part, on the migration of satellite cells from neighboring locations in the muscle to the site of injury, where their numbers expand and some of the myogenic cells then differentiate to replace the damaged muscle (111, 159, 211, 212, 263) (Fig. 8). The directed migration of satellite cells into sites of severe muscle injury depends primarily on chemotaxis that is driven by attractants released by inflammatory cells at the injury site or by attractants that are normally stored in the connective tissue in an inactive state, but are activated and released because of tissue damage. However, myogenic cells that are not derived from the satellite cell compartment can also migrate toward injury sites and participate in regeneration. Stem cells with myogenic potential appear to respond

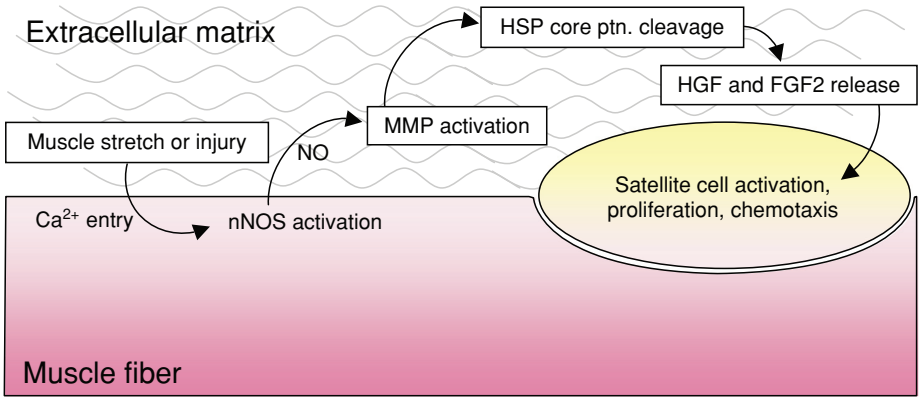

Figure 9 Schematic representation of a potential mechanism through which muscle injury could lead to satellite cell activation. Muscle membrane lesions or muscle stretching increases the calcium ion entry into muscle, producing an increase in nNOS activation. The elevated release of NO from muscle fibers can increase activation of MMP-2 present in the extracellular space. MMP-mediated cleavage of HSP core protein permits the release of HGF and FGF2 from their bound, inactive state to become active. HGF can then bind its receptor c-met that is present on the surface of quiescent satellite cells to induce their activation, proliferation, and chemotaxis. Similarly, FGF2 can binds its receptor present on the satellite cell surface to drive proliferation and chemotaxis.

to the same chemoattractants as satellite cells (e.g., reference 198). In addition, embryonic myoblasts are attracted by a similar population of chemoattractants (e.g., reference 129). These three populations of myogenic cells are grouped in the following discussion as "myogenic precursor cells" (MPCs).

Many of the potent chemoattractants for MPCs are mitogens in addition to chemoattractants, which suggests that MPC populations expand while they migrate toward the injury site. For example, platelet-derived growth factor (PDGF), a strong mitogen that can be produced by mesenchymal cells and vascular endothelial cells (54), stimulates MPC proliferation (103, 279) while also being highly chemoattractive to MPCs (256). However, both of these effects of PDGF vary with PDGF isoform. The mean number of migrating MPCs and the mean number of MPCs in a proliferation assay were increased by PDGF-BB, but not PDGF-AA (205). Similarly, FGF2 not only functions as a wound hormone to promote proliferation of MPCs, but also is chemoattractive to embryonic myoblasts, satellite cells, and myoblasts in the C2C12 cell line (205, 268). FGF2 can also play an important role in suppressing differentiation of myogenic cells while synchronizing their proliferation and migration. FGF2 maintains myogenic cells in the cell cycle so that they are unable to begin terminal differentiation (178, 214) and migrating MPCs appear to be incapable of differentiation while migrating (102, 223).

HGF also functions as a potent, perhaps the most potent, chemoattractant for myogenic cells following muscle injury. The chemoattractive capacity of HGF was indicated in early, developmental studies that showed the ectopic expression of HGF in the nervous system would lead to the formation of skeletal muscles at the ectopic site (232), which indicated that HGF production attracted myogenic cells to the site. Subsequent findings showed that HGF was a more potent chemoattractant on a molar basis than other, known muscle chemoattractants, such as PDGF, FGF2, and TGFβ (18) and that myogenic cells traveled more directly toward HGF than other chemoattractants, such as FGF2 (218).

Immune cell regulation of muscle cell migration and proliferation following injury

Stages of myogenic cell differentiation in injured muscle correlate with shifts in inflammatory cell invasion

The sequential change in patterns of expression of muscle-specific transcription factors and other developmentally regulated genes that occurs during muscle differentiation does not require the presence of other cell types. For example, when muscle fibers were isolated in culture, satellite cells on the fiber's surface showed an early, rapid upregulation of MyoD when the satellite cells entered the proliferative stage, after which the expression of myogenin and developmental myosin heavy chain (dMyHC) increased while MyoD levels declined. This shift in relative levels of expression of those transcription factors coincided with the slowing of cell proliferation and entry into the early differentiation stage of myogenesis (44, 280). This general pattern of gene expression also occurs in muscle *in vivo*, following injury (283) (Fig. 10). For example, injection of cardiotoxin into fully differentiated muscle initiated muscle injury and regeneration during which MyoD levels in satellite cells reached peak levels at about 2-day postinjury, followed by elevated myogenin expression at about 3-days following toxin injection (124, 280). Similarly, bupivicaine injections into muscle activated MyoD expression that reached peak levels at 2 to 3 days post injury, and

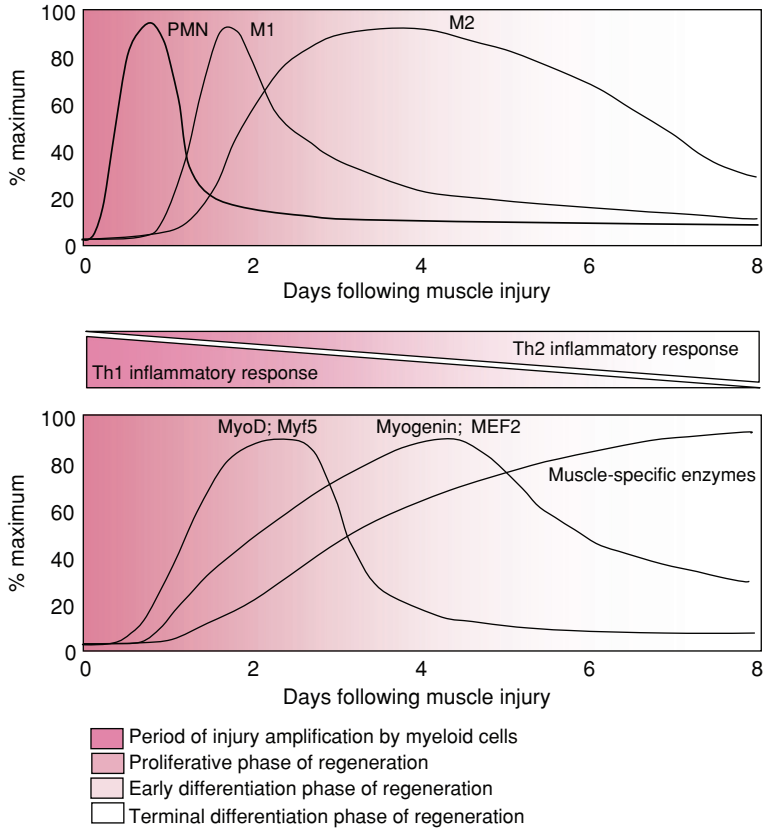

Figure 10 Diagrammatic representation of the temporal relationship between the changes in the populations of inflammatory cells in muscle following acute injury and the changes in gene expression in muscle following injury. The early stages of muscle repair following injury are characterized by a contemporaneous invasion of neutrophils (PMN) and M1 macrophages with the activation of expression of transcription factors associated with the proliferative stage of myogenesis (e.g., MyoD and Myf5). Subsequent declines in the myeloid cells associated with Th1 inflammatory response and elevated numbers of leukocytes characteristic of a Th2 response, especially M2 macrophages, occurs at the time muscle elevates expression of transcription factors associated with the early differentiation stage of myogenesis (e.g., myogenin and MEF2). As inflammation further resolves, muscle cells undergo terminal differentiation and express elevated levels of muscle-specific enzymes and structural proteins. [Adapted and modified, with permission, from reference (244)].

was followed by elevated expression of dMyHC and other proteins reflective of later stages of muscle differentiation (152).

Although the stereotypic pattern of expression of developmentally regulated genes may occur in injured muscle independent of the influence of exogenous factors, strong evidence now shows that the immune system is a powerful modulator of the proliferation and differentiation of myogenic cells in injured muscle. Further, findings suggest a strong linkage between shifts in inflammatory cell populations and changes in the stage of muscle repair and regeneration *in vivo* (Fig. 10). For example, Ly6C+/F4/80− neutrophils rapidly invade muscle following injury where they promote muscle damage and initiate the removal of cellular debris. They reach their peak concentration at approximately 24-h postinjury, and then rapidly decline (172, 217, 227, 237, 240). A population of phagocytic, CD68+/CD163−/F4/80+ macrophages (M1 macrophages) closely follows the invasion of neutrophils, reaching peak concentrations at approximately 2 days of injury, and then they also undergo a rapid reduction in numbers (175, 226). Finally, nonphagocytic, CD68−/CD163+/F480+ macrophages (M2 macrophages) invade, reaching peak concentrations at about 4-day postinjury (226), but these cells remain at elevated numbers in the injured muscle for periods as long as 2 weeks (116, 226). Notably, the activation and influx of phagocytic neutrophils and M1 macrophages coincide closely with the activation, proliferation, and migration of satellite cells, suggesting that these phagocytes may play a regulatory role in these processes. Similarly, the presence of late-invading, nonphagocytic, M2 macrophages corresponds with the period of terminal differentiation of satellite cells in injured muscle, which may reflect their function in modulating regeneration and growth of injured muscle.

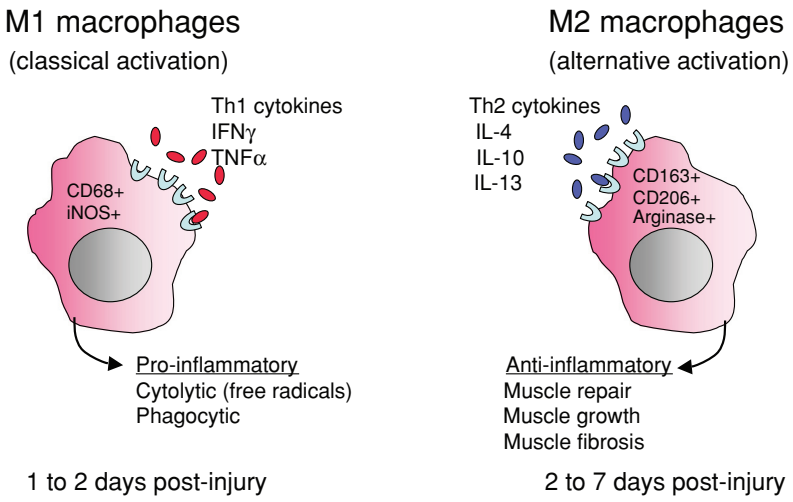

Figure 11 Diagram of alternative fates of macrophages activated to the M1 phenotype by Th1 cytokines or to the M2 phenotype by Th2 cytokines. In addition to promoting the M2 phenotype, IL-10 can also deactivate the M1 phenotype. Following muscle injury, M1 macrophages dominate the inflammatory infiltrate between 1 and 2 days postinjury, when they can promote further analysis of muscle membranes by free radical production and remove cellular debris by phagocytosis. The M1 macrophages are they superceded by the M2 population between days 2 and 4 postinjury. M2 macrophages attenuate inflammation by deactivating M1 macrophages. They also promote muscle repair, growth, differentiation, and fibrosis.

Are Th1 cytokines or chemokines important for regulating the proliferation or migration of myogenic cells to sites of muscle injury?

M1 macrophages and neutrophils are rich sources of Th1 cytokines that can promote myeloid cell activation and chemotaxis to sites of tissue injury; however, many of these cytokines also affect the proliferation and migration of muscle cells. Extensive research now shows that Th1 cytokines play in important role in regulating muscle repair and regeneration by influencing the proliferation and chemotaxis of muscle cells, in addition to their better-characterized role in regulating immune responses. Among the many Th1 cytokines that are elevated in injured muscle during the proliferative stage of myogenesis, interferon-gamma (IFNγ) and tumor necrosis factor-alpha (TNFα) appear to play especially important roles in influencing satellite cell proliferation or migration. However, other small, chemoattractant molecules, called chemokines, that are released by inflammatory cells can also significantly affect early stages of muscle regeneration and appear to assert their effects most prominently during the early inflammatory response.

- Interferon-gamma (IFNγ).

IFNγ is a prototypical Th1 cytokine and powerful activator of neutrophils and the M1 macrophage phenotype. Although IFNγ is generally viewed as a product of only natural killer cells and T cells, M1 macrophages can be induced to express high levels of IFNγ in innate immune reactions, so that IFNγ can serve an autocrine role driving M1 macrophage activation (163) (Figs. 11 and 12). Furthermore, IFNγ stimulation can enhance the responsiveness of neutrophils to chemotactic cytokines (21), thereby potentially increasing their invasion into injury sites. Thus, the elevated levels of IFNγ expression that accompany acute (35) and chronic (258) muscle injuries have the potential to amplify the innate immune response, and potentially to exacerbate muscle damage that occurs during the early Th1 inflammatory response.

IFNγ may also have direct effects on muscle cells to influence repair and regeneration following injury that may be beneficial to the repair process (Fig. 12). Rat myotubes express the IFNγ receptor (IFNγR) at low levels constitutively, but receptor expression can be greatly elevated by exposure to TNFα, IFNγ, and endotoxin (287) suggesting that IFNγ-mediated signaling would be enhanced in muscle cells in injured tissue. IFNγ treatments cause significant increases in MPC proliferation, without affecting fusion rates (108), but can also influence the survival of myotubes by blocking apoptosis. When myotubes *in vitro* are treated with TNFα, apoptosis can be induced, leading to loss of muscle, but IFNγ can prevent TNFα-induced muscle apoptosis by downregulating the TNFα receptor, TNF-R2 (248). Thus, elevations of IFNγ in muscle following acute injury would be expected to stimulate muscle repair and regeneration by increasing the proliferation and survival of muscle cells.

The function of IFNγ as both a proinflammatory molecule and a muscle mitogen raises the issue of whether IFNγ serves a beneficial or negative role in muscle repair and regeneration (Fig. 12). This question was addressed using a cardiotoxin-injection, acute muscle injury model, which showed that expression levels of IFNγ were rapidly elevated in injured

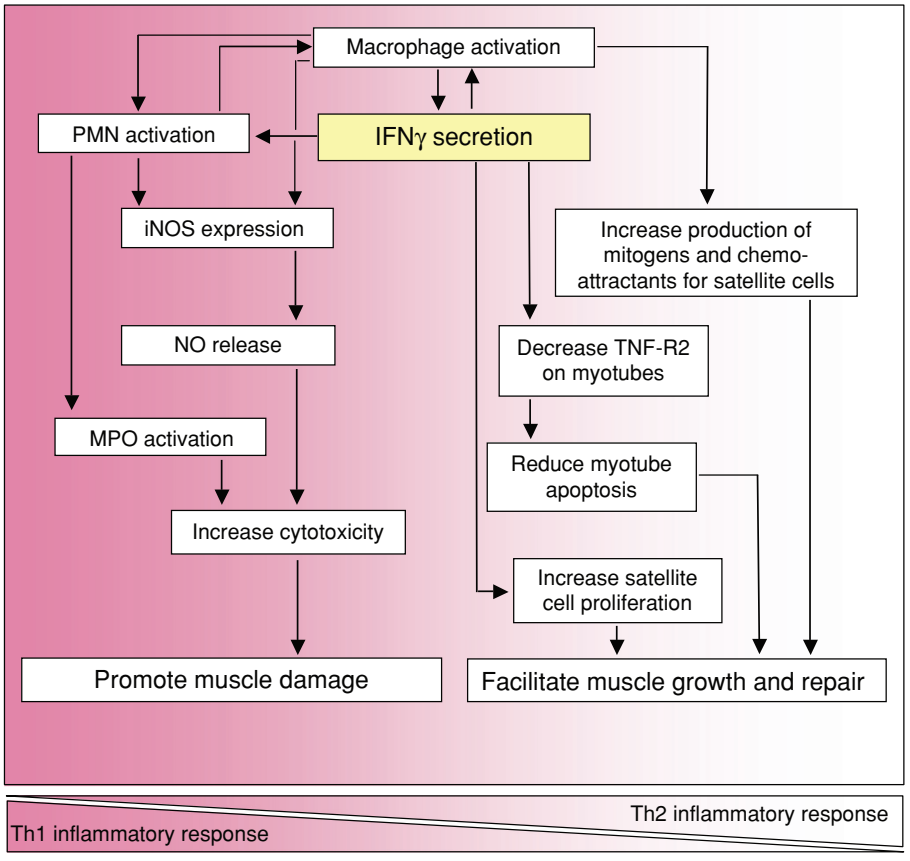

Figure 12 Simplified summary diagram of the competing roles played by IFNγ in muscle injury and repair. IFNγ can be expressed and secreted by M1 macrophages, further driving monocytes to the M1 macrophage phenotype and also activating neutrophils. IFNγ stimulation of neutrophils and M1 macrophages elevates their expression of constitutively active iNOS, increasing the production of NO to levels that can be cytolytic. Activation of neutrophils by IFNγ can also increase their production and release of MPO, which can also promote cytotoxicity and muscle damage. These functions of IFNγ appear to be most important during the Th1 inflammatory response. IFNγ can also act directly on muscle cells to increase their proliferation, attract them to sites of tissue damage and inhibit apoptosis. These functions of IFNγ appear to be most important during the Th2 inflammatory response while muscle repair and regeneration proceed.

muscle, reaching a peak at 5-day postinjury, and then declining (35). Several observations suggested that increased expression of IFNγ could have a potentially beneficial role in muscle repair by promoting MPC proliferation and muscle regeneration. In particular, administration of neutralizing antibodies to the IFNγR to mice with injured muscles caused significant reductions in the numbers of central-nucleated fibers, declines in the numbers of proliferative cells that incorporated bromodeoxyuridine (BrDU), and reductions in the number of cells that expressed MyoD (35). Although these treatment effects could reflect a reduction in muscle repair or regeneration when IFNγ signaling was impaired, the data are also consistent with the possibility that IFNγ promotes muscle injury instead of regeneration. Because IFNγ is a strong activator of neutrophils and M1 macrophages, and those cells promote muscle damage following acute injury (e.g., reference 190), reductions of IFNγ signaling by administration of neutralizing antibodies to the IFNγR would be expected to reduce the numbers and activation of cytotoxic myeloid cells. Reductions in their numbers and activity would similarly yield less muscle regeneration that would be reflected in fewer central-nucleated fibers, as a consequence of less muscle damage, and fewer proliferative cells, as a consequence of less inflammation.

- TNFα

TNFα, another Th1 cytokine that is highly expressed by M1 macrophages, is a complex regulatory molecule with the potential to affect muscle repair and regeneration through its actions as a mitogen and motogen for skeletal muscle cells. However, similar to IFNγ-mediated effects following muscle injury, TNFα also has the capacity to promote muscle damage (Fig. 13). Following acute injuries, TNFα expression in muscle reaches its peak at about 24-h postinjury, which coincides with the invasion by neutrophils and M1 macrophages and the increase in secondary muscle damage caused by myeloid cells (262). Because much of the myeloid-cell-mediated damage to

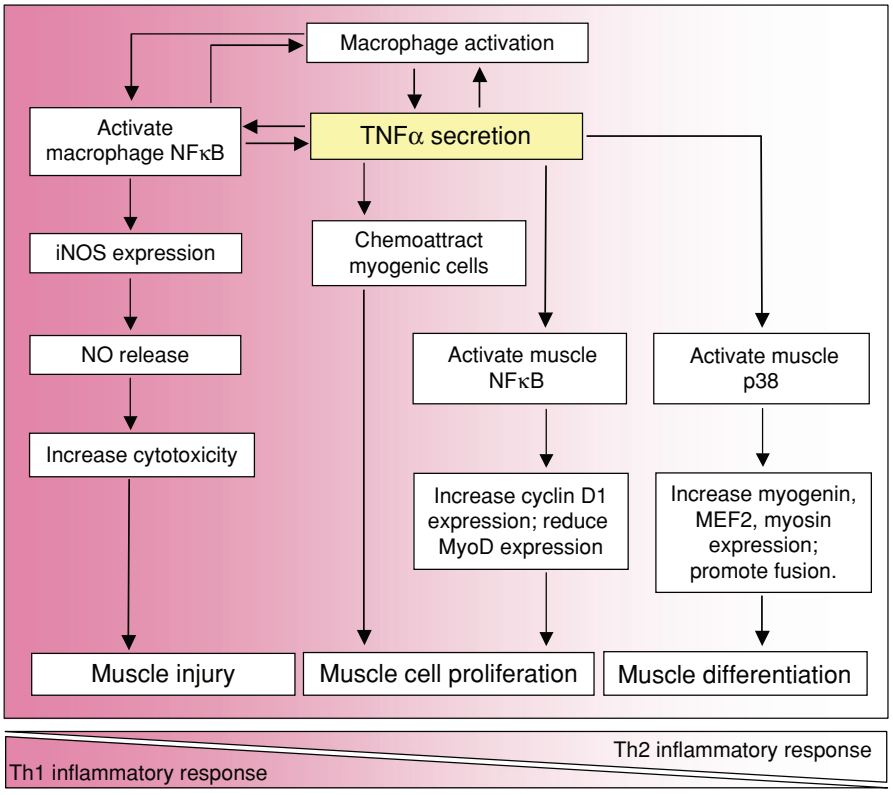

Figure 13 Simplified summary diagram of the competing roles played by tumor necrosis factor-alpha (TNFα) in muscle injury and repair. TNFα secreted by M1 macrophages can activate NFκB in monocytes and macrophage to drive them to an M1 macrophage phenotype. NFκB activation promotes the expression of iNOS in macrophages, thereby increasing the production of NO to levels that can be cytotoxic. TNFα also function as a chemoattractant to myogenic cells, drawing them to sites of muscle injury. These functions of TNFα appear to be most important during the Th1 inflammatory response. TNFα can also activate NFκB in proliferative, satellite cells to increase their proliferation and suppress their differentiation. TNFα can also activate signaling via p38 in satellite cells to promote their differentiation and fusion. These latter functions of TNFα are expected to be most important during the Th2 inflammatory response when muscle repair and regeneration occur.

muscle fibers is caused by iNOS-derived NO (171, 258) and because TNFα stimulation of M1 macrophages can elevate their expression of iNOS and promote their ability to lyse muscle fibers (258), TNFα activation of inflammatory cells has been associated with promoting muscle damage.

Despite the potential for TNFα to promote damage of injured muscle through its activation of the Th1 inflammatory response, it also has the potential to promote repair and regeneration through its direct actions on muscle cells (Fig. 13). *In vitro* studies have shown that TNFα is a chemoattractant for MPCs (249) and that the chemoattraction of muscle stem cells toward M1 macrophages could be significantly inhibited by neutralizing antibodies to TNFα (140), suggesting that the migration of satellite cells and stem cells to sites of muscle injury and inflammation could be driven in part by TNFα.

TNFα can also influence muscle repair and regeneration by modulating the proliferation and differentiation of satellite cells in injured tissue. Application of TNFα to MPCs *in vitro* increased their proliferation (134) and inhibited their fusion (90, 122, 123), indicating that TNFα can maintain satellite cells in a proliferative state, while they migrate to the site of muscle injury to participate in tissue repair. *In vivo* observations also indicate a role for TNFα in maintaining muscle cells in the cell cycle. For example, TNFα null mutants and TNFα receptor mutants showed lower levels of MyoD and MEF-2 expression than wild-type controls following acute muscle injury (34, 263). In addition, the systemic elevation of TNFα caused by expression of a lung-specific, TNFα transgene reduced the expression of dMyHC in mice subjected to hindlimb unloading followed by 3 to 5 days of muscle reloading (121). Because dMyHC expression is normally increased during the early and terminal stages of myogenesis, suppression of dMyHC could reflect a role for TNFα in retaining satellite cells in the proliferative stage and delaying transition to the early differentiation stage of myogenesis.

Many of the effects of TNFα on muscle cells during the early stages of muscle repair may result from activation of NFκB in muscle cells. NFκB is a transcription factor that is retained in the cytoplasm in an inactive form when it is bound to endogenous inhibitors. Stimulation of cells with

Th1 cytokines, such as TNFα or IL-1, causes inactive NFκB to disassociate from its inhibitor and then translocate to the cell nucleus where it can induce the transcription of Th1 cytokines and other pro-inflammatory molecules including iNOS, TNFα, IL-1, and CCL2 (161). Thus, TNFα-mediated activation of NFκB can generate positive feedback that can exacerbate inflammation and increase the production of enzymes that drive cytolytic processes in injured muscle. However, NFκB also increases the expression and stability of cyclin D1 (8, 90, 97), which can increase cell proliferation and inhibit differentiation. NFκB activation in muscle can also increase degradation of MyoD protein (123), which would negatively affect muscle differentiation and tend to retain MPCs in the proliferative stage of myogenesis. However, at least some of the inhibitory effects of NFκB on muscle differentiation appear to occur through processes that do not involve direct interactions of NFκB with the promoters of muscle-specific genes. Instead, the suppressed expression of many myofibrillar genes by NFκB is attributable to its binding to the promoter of the transcriptional repressor YY1, leading to increased expression of YY1, which then represses the expression of multiple genes that are upregulated in late differentiation of muscle cells (260).

TNFα levels remain elevated in injured skeletal muscle long after myogenic cells have exited the proliferative stage of myogenesis and entered the early-to-terminal stages of differentiation, suggesting an additional regulatory role for TNFα in later stages of muscle regeneration (Fig. 13). However, the prodifferentiation effects of TNFα on muscle occur through signaling through pathways that are independent of NFκB activation. TNFα can activate p38 kinase in muscle (195), and activated p38 can promote muscle differentiation. This effect has been demonstrated in skeletal muscle cells *in vitro* where inhibition of p38 reduced the expression of myogenin, MEF2 and myosin light chain kinase and inhibited muscle cells from fusing to form myotubes (286). Furthermore, increased activation of p38 increases the activity of MyoD (286), an effect that opposes the influence of TNFα activation of NFκB in muscle.

Although p38 can promote myogenesis by increasing MyoD activity, its phosphorylation of other, muscle-specific transcription factors can produce outcomes that may either compete or synergize with the promyogenic effects mediated through MyoD. On one hand, p38 activation can further promote myogenesis by phosphorylation and activation of MEF-2 (91, 286). However, p38 phosphorylation of myogenic regulatory factor-4 (MRF-4) deactivates MRF-4, preventing its transactivation of muscle-specific genes such as desmin and skeletal α-actin *in vitro* (228). Similarly, perturbations of MRF4 expression or activity *in vivo* disrupt normal muscle regeneration (185).

- Chemokines.

Although chemokines are primarily associated with their role in attracting leukocytes to sites of injury and inflammation, their functions are more complex and are not limited to immune cells. In particular, these small, leukocyte-derived molecules can play important roles in modulating the chemotaxis, activation, and differentiation of MPCs in injured muscle *in vivo*. Among the large, complex family of chemokines and their receptors, chemokines in the CC class and their receptors are expressed most highly in injured or diseased muscle, which may reflect a role in modulating muscle repair or regeneration. CC chemokines receive their designation in the CC class because they share the common structural feature that there are two adjacent cysteines near their amino terminus. This feature distinguishes them from other chemokine classes in which there is only one cysteine near the amino terminus (C chemokines) or multiple cysteines separated by a variable number of noncysteines (CXC or CX_3C chemokines).

Perturbations of CC cytokine signaling in injured muscle can significantly influence muscle regeneration. Following acute injury, expression levels of CC ligand-2 (CCL2), CCL3, and CCL4 and their shared receptors, CCR2 and CCR5, increase rapidly and are sustained through the regenerative process in muscle (264, 266). However, current findings are only supportive of a role of CCL2/CCR2 signaling in muscle regeneration. Although null mutants for CCL2 showed slower functional recovery following muscle injury than occurred in wild-type mice, the rate of muscle force recovery following injury was not affected by ablation of CCR5 (264, 266). Furthermore, the investigators showed the presence of CCR2 on the surface of myogenin-expressing muscle cells in the injured muscle (264) indicating that the proregenerative effects of CCL2 in injured muscle could result from direct actions on MPCs. However, CCR2 was also expressed by endothelial cells, macrophages, and dendritic cells in the injured muscle (264), so that part of the CCL2 effect on regeneration could be mediated by those other cell types.

Other findings show that signaling through CCL2/CCR2 in injured muscle has direct effects on the inflammatory process that may underlie the defects in regeneration that are caused by ablating this signaling pathway. For example, macrophage invasion in toxin-injected muscles was significantly reduced by null mutation of CCR2, although neutrophil invasion was amplified (175). However, revascularization of the lesion site was slowed in CCR2 null mice (175), so that part of the regenerative defect may be attributable for deficient blood supply. The mutation also produced a slowing of the growth of regenerative, central-nucleated fibers at the injury site (230). Importantly, bone-marrow transplantation from wild-type donors into recipients receiving myeloablative irradiation before toxin-injection restored normal levels of muscle invasion by macrophages and reversed defects in muscle regeneration that were assessed by assaying the size of regenerative, central-nucleated fibers (230). Together, these findings suggest that a significant portion of the proregenerative effects of CCL2/CCR2 signaling are mediated through the myeloid compartment.

Although muscle repair and regeneration are apparently promoted by chemokine signaling through myeloid cells that are most likely to be macrophages, *in vitro* observations indicate that direct actions of chemokines on muscle cells themselves can also affect muscle cell function. Myoblasts constitutively express CCR1, CCR2, CCL4, and CCR5 and stimulation of myoblasts with CCL2 or CCL4 increases their proliferation (281). Because myoblast stimulation with CCL2, CCL3, or CCL4 also induces phosphorylation and activation of ERK1/2 (281) and ERK1/2 activation occurs in a pathway through which some growth factors stimulate cell proliferation, the findings suggest that CCL2 activation of the ERK1/2 pathway in myogenic cells may contribute to muscle regeneration.

Deactivation of the Th1 inflammatory response and its potential role for satellite cell transition from the proliferative stage to the early differentiation stage in muscle repair and regeneration

Entry of myogenic cells to the stages of early and terminal differentiation requires their exit from the cell cycle. Because many of the mitogenic substances that are responsible for driving satellite cell proliferation after injury are also chemoattractants, the exit of satellite cells from the cell cycle coincides with the ending of their migration. Thus, decline in the production or release of mitogens and motogens provides an important means for controlling both the onset and site of myogenic cell differentiation following injury. However, neutrophils and M1 macrophages are major sources for Th1 cytokines and chemokines that promote satellite cell proliferation and migration but Th1 cytokines can further drive the activation of neutrophils and M1 macrophages to produce Th1 cytokines. This positive feedback would tend to retain satellite cells in the proliferative stage if the Th1 inflammatory response were perpetuated. Thus, attenuation of the Th1 inflammatory response and its role in driving the proliferative stage of myogenesis would require a negative regulator to deactivate neutrophils and M1 macrophages and thereby facilitate the transition of satellite cells to terminal differentiation. Accumulating evidence suggests that phagocytosis of debris by myeloid cells may provide a negative signal that attenuates Th1 activation, and thereby contributes to transition of muscle cells to early differentiation.

Removal of tissue debris by phagocytes is a common, early occurrence following muscle injury, although the initial signals that activate phagocytosis by myeloid cells in injured muscle are not known with certainty. However, ligation of complement receptors on neutrophil or macrophage surfaces is likely a major contributor to myeloid cell activation in injured muscle because complement activation occurs soon after muscle injury (69, 120, 186, 207, 220) and complement receptors are expressed constitutively on the surfaces of neutrophils and macrophages, especially proinflammatory M1 macrophages. Upon activation, neutrophils can then further amplify the phagocytosis of debris by M1 macrophages. For example, oxidative modification of low-density lipoproteins (LDLs) by MPO of neutrophil origin enhances LDL binding to CD68 on the surface of M1 macrophages (288) and ligation of CD68 enhances phagocytosis by macrophages and increases their production of proinflammatory, Th1 cytokines (179, 196, 255). The elevated production of proinflammatory cytokines can then further promote activation of M1 macrophages. Thus, phagocytosis of debris by neutrophils may potentially initiate a positive feedback that propagates the inflammatory response that is dominated by phagocytes, Th1 cytokines, and M1 macrophages.

Phagocytosis also has the potential to deactivate the inflammatory response by shifting M1 macrophages to an anti-inflammatory, M2 macrophage phenotype. *In vitro* studies demonstrated that M1 macrophages reduced their secretion of the Th1 cytokine TNFα and increased production of the Th2 cytokine TGFβ after phagocytosis of muscle debris, which suggested that phagocytosis could induce a shift toward a M2 phenotype (6). However, there is an additional layer of complexity in the regulation of macrophage phenotype switching by phagocytosis: whether the phenotype switch occurs depends on the composition of the debris ingested. For example, if the debris were generated by neutrophil lysis, phagocytic macrophages would greatly increase IL-10 production, which reflects a switch to a M2 phenotype (63). The elevation in IL-10 production would have further functional importance in regulating the production of Th1 cytokines that could drive muscle cell proliferation because IL-10 is a powerful deactivator of the M1 phenotype. In contrast, debris produced by lymphocyte lysis does not induce an increase in IL-10 production by macrophages (63). This suggests that phagocytosis may induce phenotype switching in macrophages as part of the innate immune response, which would occur in response to acute muscle injury, but not in the context of an acquired immune response. Other findings further suggest that the role of phagocytosis in macrophage phenotype switching may differ according to whether phagocytosis occurs during innate or acquired immune response. Phagocytosis of apoptotic neutrophils by M1 macrophages suppressed expression of IL-1β and TNFα and increased expression of TGFβ by the macrophages, indicating a shift toward an M2 phenotype (64). However, if the apoptotic neutrophils were opsonized with immunoglobins to CD45, their phagocytosis by macrophages did not produce a shift in macrophage phenotype (64). These observations suggest that phagocytosis of apoptotic neutrophils in the context of an innate immune response can drive macrophage phenotype switch, which agrees with *in vivo* observations in injured muscle. For example, the peak of apoptosis and phagocytosis of inflammatory cells in the rodent hindlimb unloading/reloading model of muscle injury and repair occurs 2 days after the onset of muscle reloading (243) which coincides with the shift from M1 to M2 macrophage phenotypes.

Is phagocytosis of debris in injured muscle necessary for successful muscle regeneration?

Despite the potential, beneficial roles of phagocytosis in regulating macrophage phenotype during inflammation and for clearing tissue debris in preparation for muscle regeneration, not all *in vivo* findings indicate that phagocytosis is an essential component of muscle repair and regeneration following injury. However, differences in interpretation of the importance of phagocytosis in muscle regeneration may reflect differences in the injury model used, the method of reducing phagocytic cell populations, and the assays chosen to assess repair and regeneration. For example, depletion of phagocytic cells was achieved in one investigation by *in vivo* administration of mice with RB6-8C5 prior to muscle injury by toxin injection (237). RB6-8C5 is a monoclonal antibody that binds Ly6C and Ly6G which are expressed on the surface of neutrophils and monocytes; binding RB6-8C5 would selectively opsonize neutrophils and monocytes, leading to their selective depletion. In a second investigation, phagocytes were depleted by generation of a mouse strain that expressed a transgene encoding the diptheria toxin (DPT) receptor gene driven by the CD11b promoter, and then treating the mice with DPT before muscle injury (6). In this latter model, cells that normally express CD11b would express the transgene, leading to their binding of DPT and cell death. Neutrophils, monocytes, and macrophages express CD11b and are all potential targets in this strategy. In both studies, reduction of myeloid cell numbers from mice prior to muscle injury by toxin injection slowed the removal of cellular debris and reduced some indices of muscle regeneration (6, 237). In both models, there were fewer central-nucleated fibers in the muscles of mice in which the numbers of myeloid cells and the extent of phagocytosis were decreased (6, 237) which could reflect a reduction in regeneration as a consequence of diminished phagocytosis. However, both neutrophils and M1 macrophages are cytolytic cells that can promote muscle damage following acute injury, so that their depletion could also decrease muscle damage following toxin injection. For example, rabbit muscle injury that resulted from eccentric contractions was significantly reduced if the animals were treated with a function-blocking anti-CD11b that decreased the numbers of phagocytic neutrophils that invade the muscle following injury (26). Antibody blocking CD11b prior to puncture injuries of muscle similarly reduced the number of phagocytic leukocytes that invade the injured muscle and reduced muscle injury (285). Although not assayed, this reduction of injury would be expected to be reflected in a smaller number of central-nucleated fibers in the muscle during the regenerative stage following injury. Thus, the extent to which reductions of regeneration in injured muscle reflect impaired regeneration that is attributable to leukocyte depletions or reflects less muscle damage caused by leukocytes remains uncertain.

Other investigations raise questions concerning the importance of phagocytosis in the process of muscle repair and regeneration. Depletion of phagocytes from mice by injecting liposome-encapsulated clodronate prior to freeze injury of the muscle slowed the removal of cellular debris, reflecting a reduction in phagocytosis (229). However, the levels of MyoD and myogenin expression in muscle at 3-day postinjury did not differ between clodronate-treated and nontreated mice, which could be interpreted as evidence that regeneration did not differ according to these particular indices. Nevertheless, other indices of regeneration could possibly reveal a treatment effect in the phagocyte-depleted mice. In particular, perhaps the effects of reductions in phagocytosis on muscle regeneration would not be manifest until terminal differentiation of the regenerative cells, which could be reflected in numbers of central-nucleated fibers but not in levels of MyoD or myogenin expression.

M2 macrophage function in the regeneration and growth of injured muscle

The coordinated transition of macrophages from an M1 to an M2 phenotype while injured muscle transitions from the proliferative stage to the early stage of differentiation may be attributable, in part, to the capacity of M2 macrophages to promote later stages of muscle repair and regeneration (Fig. 11). Early immunohistological observations noted the close apposition between M2 macrophages and regenerative fibers in injured muscle, as well as the coinciding increase in M2 macrophage numbers and muscle regeneration (226). More recently, experimental findings have supported this likely role for M2 macrophages in muscle regeneration using the unloading/reloading model in which M2 macrophages invade muscle in large numbers between days 2 and 4 of reloading while M1 macrophages decline in number (245). Depletion of macrophages between days 2 and 4 of reloading prevented the repair of injured muscle cell membranes that occurred in nondepleted mice and attenuated the decrease in dysferlin expression and arrested the increase in central-nucleated fibers that normally occurred between days 2 and 4 or reloading. These findings indicated an important role for macrophages in those aspects of muscle's response to injury. Furthermore, growth and differentiation during reloading were disrupted. Between 2 and 4 days of reloading, muscle normally shows a decline in MyoD expression and an increase in myogenin expression that reflects a transition from the proliferative to the early differentiation stages of myogenesis. However, macrophage depletion prevented those changes in transcription factor expression in the reloaded muscle. Furthermore, increases in muscle fiber cross-sectional area that were caused by fiber growth during 2 to 4 days of reloading were similarly prevented by the depletion of late-invading macrophages (245). Although these findings show important roles for macrophages that are present in 2 to 4 days of reloaded muscle in promoting muscle fiber repair, differentiation and growth, the data are not sufficient to prove that the effects were specifically mediated by M2 macrophages because the depletion protocol may also have affected M1 macrophages that remained in the muscle.

Perturbations in the numbers or activity of late-invading myeloid cells in other models of muscle injury also support a role for M2 macrophages in promoting muscle growth and regeneration. Null mutation of either CCR2 or CCL2 produced tremendous reductions in the numbers of macrophages present in muscle that was injured by either ischemia (42, 217) or cardiotoxin injection (175), and these reductions of macrophages at those stages of muscle repair and regeneration were accompanied by delayed appearance of regenerative muscle fibers and slower muscle fiber growth at the site of muscle repair. Furthermore, the investigators discovered that these effects of CCR2 or CCL2 mutation on muscle regeneration were mediated through the myeloid compartment. When wild-type bone marrow was transplanted into irradiated, CCR2-mutant mice prior to muscle injury, macrophage invasion was restored along with muscle growth following muscle injury by toxin (230). However, once again, the data are not definitive for a specific role for M2 macrophages in muscle regeneration since other myeloid cells would also be present in relatively low numbers at this stage of muscle repair and regeneration and they could also be affected by the treatment.

Chronic muscle injuries

The causes and consequences of muscle injury are best understood for acute muscle injuries that are caused by trauma or exercise. However, some diseases can cause chronic muscle injuries in which the pathology can persist for decades and involve repeated rounds of damage, inflammation, regeneration, and repair. Although many of the mechanisms that underlie the response of muscle to chronic injury are similar to the mechanisms that mediate repair and regeneration follow acute injury, the recurring damage can lead to a more complex repair response and can produce a more complex inflammatory response that can have long-term effects on the viability and function of the muscle.

Dystrophin-deficient muscle provides the best-studied model of chronic muscle damage, especially in investigations using the *mdx* mouse model of DMD. Dystrophin is a sarcolemma-associated, cytoskeletal protein that provides mechanical stability to the muscle cell membrane and secures a transmembrane complex of signaling molecules at the muscle cell surface (98, 177, 189). Null mutation of the dystrophin gene produces a weaker cell membrane and leads to a secondary reduction (176), but not loss, of proteins in the transmembrane complex. Because the muscle membrane is weaker, membrane lesions occur more rapidly, which leads to the unregulated translocation of ions and molecules across the cell membrane. As a consequence, many of the pathogenic events at the early stages of pathology of dystrophin-deficient muscle resemble those encountered following acute muscle injury. The unregulated influx of calcium ions into the damaged muscle fibers activates calcium-dependent proteases, complement activation occurs, chemotactic molecules are released or activated, an innate immune response is initiated, satellite cells are activated and muscle repair and regeneration follow. Just as in acutely injured muscle, much of this pathogenic sequence is directly or indirectly attributable to damage to the sarcolemma.

Muscle membrane damage and repair in chronic muscle injuries

Empirical evidence provides strong support for the conclusion that membrane damage in dystrophin-deficient muscle fibers can be caused by mechanical stresses placed on the muscle cell membrane and that this is a primary defect in the disease (189). This interpretation is further supported by studies that have related changes in the *mdx* pathology to changes in the expression of the dystrophin-homolog, utrophin, to *mdx* pathology. Utrophin is normally expressed in myotubes, neuromuscular junctions, and MTJs in *mdx* muscle (126, 173, 177). Following an initial bout of severe muscle damage that peaks at about 4 weeks of age in *mdx* mice, *mdx* muscles experience a remarkable period of rapid regeneration that coincides with an increase in utrophin expression (4, 45, 47, 126). During this period of regeneration, utrophin is expressed in some fibers all along the surface membrane, which is sufficient to reduce membrane damage in mechanically stressed muscles and supports the interpretation that regeneration is facilitated by repair of mechanical defects enabled by increased utrophin expression. Experimental manipulations of utrophin expression also greatly increase the strength of *mdx* muscle membranes and reduce pathology. For example, overexpression of utrophin in *mdx* muscle that is driven by an utrophin transgene can essentially prevent muscular dystrophy (51, 194, 246). In addition, driving expression of native utrophin to higher levels by treatments of *mdx* mice with an activator of peroxisome proliferators-activated receptor greatly reduces muscle pathology (157).

Muscle membrane damage by myeloid cells in chronic muscle injuries

Although mechanical weakness is a primary defect in the pathophysiology of dystrophin-deficient muscles, numerous investigations indicate that inflammatory cell involvement in the disease underlies as much as 50% to 80% of the membrane damage that occurs. For example, 4-week-old *mdx* mice that had been depleted of macrophages starting at 6 days of age until 4 weeks of age showed an 80% reduction of fibers showing membrane lesions (270). Similar reductions of muscle membrane lesions occurred in *mdx* mice in which inflammation was reduced by treating with glucocorticoids (272), or null mutation of MMPs that can activate the inflammatory response (133) or by treating with metalloproteinase inhibitors that reduce inflammation (118). In addition, pharmacological or genetic interventions that inhibited NFκB signaling (1) or treatment of *mdx* mice with the free radical scavenger N-acetylcysteine, which reduces NFκB activation and inflammation (275), caused reductions in muscle fiber

membrane damage that were quantitatively similar to those achieved by macrophage depletion. Together, these findings show a basic similarity between the roles played by inflammatory cells in promoting muscle damage following either acute injury or during chronic disease. In the case of *mdx* muscular dystrophy, the mechanically induced fiber damage is amplified 2- to 5-fold by inflammatory cells, especially macrophages.

Just as occurs in acutely injured muscle, the myeloid cells that initially invade *mdx* muscle at the onset of inflammation are primarily neutrophils and iNOS-expressing, phagocytic, M1 macrophages (258). Also like acutely injured muscle, neutrophils and M1 macrophages amplify *mdx* muscle damage by the metabolism of arginine by iNOS to produce cytotoxic levels of NO (258). However, unlike acutely injured muscle, the neutrophil and M1 macrophage influx is accompanied by contemporaneous invasion by M2 macrophages that are more specifically of the M2a phenotype (Fig. 14) (258), which is characterized by elevated expression of IL-4, CD206, and arginase (84, 146). M2a macrophages are typically associated with wound healing in other injured tissues, but they also function in the early stages of *mdx* pathology to modulate the cytotoxicity of M1 macrophages because the arginase in M2a macrophages competes with the iNOS in M2a macrophages for their common substrate, arginine (Fig. 15). This competition can decrease NO production by iNOS, reduce cytotoxicity and diminish muscle cell lysis by M1 macrophages.

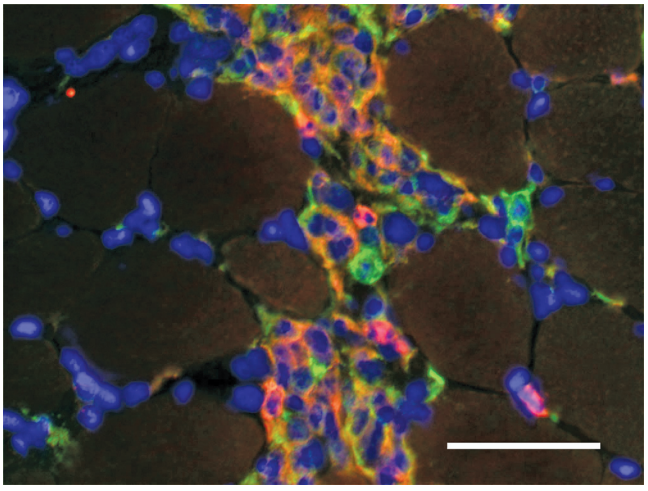

Figure 14 Immunohistochemistry of an inflammatory lesion in muscle in the *mdx* mouse model of Duchenne muscular dystrophy. This lesion contains pro-inflammatory, M1 macrophages (red) that can promote muscle damage, as well as anti-inflammatory, M2 macrophages (orange), that can affect regeneration and fibrosis. The lesion also contains muscle satellite cells (green) whose proliferation and differentiation can be modulated by the neighboring macrophages. The antibodies used for labeling were anti-F4/80 (red fluorophore) which binds all macrophages and anti-CD206 (green fluorophore) that binds M2 macrophages and satellite cells. Hoechts labeling (blue) shows nuclei. Bar = 50 μm. [Reproduced, with permission, from reference (244)].

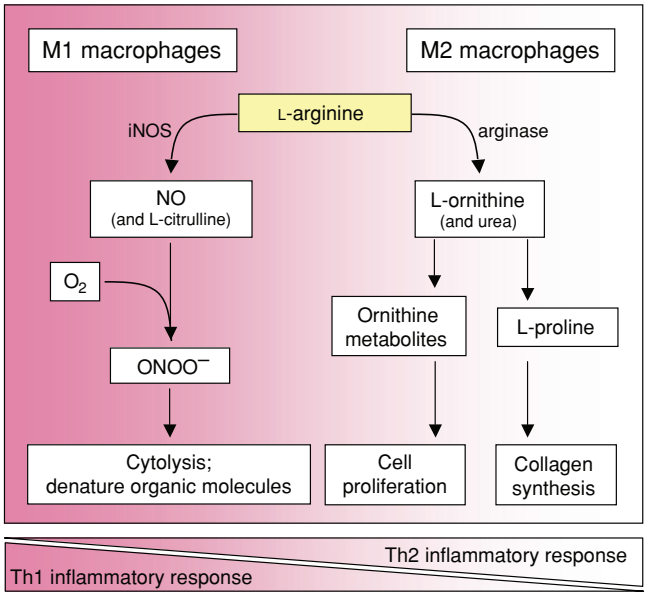

Figure 15 Diagram of pathways for arginine metabolism that contribute to muscle injury, repair, and fibrosis. Arginine is a conditionally essential amino acid that is responsible for substrate limitation of NOS activity and arginase activity under conditions of rapid growth during development or during tissue repair following injury. Thus, NOS and arginase can compete for substrate in inflamed tissues following injury. Arginine metabolism by iNOS drives the production of NO to high levels that contribute to the formation of more toxic free radicals such as peroxynitrite ($ONOO^-$) that may contribute to the formation of membrane lesions and to denaturing of proteins, lipids, and nucleic acids. Arginine metabolism by this pathway would be amplified during the Th1 inflammatory response to injury. However, arginine is also metabolized by arginase present in M2 macrophage to yield its hydrolysates L-ornithine and urea. L-ornithine is then further metabolized to yield L-proline that is necessary for synthesis of collagen and to yield other metabolites that drive the proliferation of cells, including fibroblasts. Thus, the amplification of this pathway of arginine metabolism in M2 macrophage during the Th2 inflammatory response would contribute to fibrosis and would healing.

Regulation of the inflammatory cell phenotype in chronic muscle injuries

The onset of rapid regeneration in *mdx* muscle also coincides with a transition in macrophage phenotypes from the early stage dominated by M1 macrophages and M2a macrophages to a later stage in which M2a and M2c populations prevail in the regenerative muscle (258). M2c macrophages express elevated levels of IL-4, IL-10, and CD163, which may play important roles in regulating the pathology of muscular dystrophy. IL-4 treatment of macrophages can shift them from a cytotoxic M1 phenotype to the M2c phenotype that promotes tissue repair (83,84) which has the potential to aid in the shift of *mdx* muscles from the acute necrotic stage at 4 weeks of age to the subsequent, regenerative phase. However, IL-4 also acts directly on muscle cells and can promote their fusion and growth (99) which may play an important but unexplored role in regulating the course on pathology in *mdx* mice.

IL-10 production by M2c macrophages can also play an immunomodulatory role in injured muscle by deactivating M1

macrophages and decreasing their cytotoxicity. IL-10 treatment of M1 macrophages *in vitro* decreases the expression of iNOS and this is associated with a reduction of macrophage lysis of muscle cells (259). *In vivo* findings also support the conclusion that IL-10 deactivation of M1 macrophages reduces muscle cell injury. Macrophages that were isolated from *mdx* mice that were null mutants for IL-10 were more highly cytolytic than macrophages from *mdx* muscles of mice that expressed IL-10, and IL-10 null/*mdx* mice showed more muscle membrane lesion and less strength and endurance than *mdx* mice that expressed IL-10 (259). IL-10 may play a further important regulatory role in the response of muscle to damage by promoting the switch of macrophages from the M1 phenotype that dominates the early cytolytic stage of *mdx* muscle inflammation to the later regenerative stage. IL-10 stimulation of macrophages increased their phagocytosis and their expression of CD163 (28, 259), events that may be functionally related; CD163 is a scavenger receptor and its ligation can increase phagocytosis (117, 209). Thus, the induction of phagocytosis by IL-10 has the potential to promote macrophage transition to the M2 phenotype because phagocytosis can activate the M2 phenotype, at least *in vitro* (6). Furthermore, phagocytosis-induced switching to the M2 phenotype increased the production of IL-10 by macrophages (6), which would further promote the phenotype switch, and increase expression of the Th2 cytokine, TGFβ (6). The increase in TGFβ could further promote repair by modulating the proliferation and differentiation of MPCs (2, 3, 93). However, TGFβ is also a potent, profibrotic cytokine that can increase connective tissue production by fibroblasts, which can facilitate wound repair.

Fibrosis in chronically injured muscle

Increased connective tissue production is a beneficial component of the immune response to acute tissue damage that is necessary for healing. However, chronic muscle injuries can lead to excessive accumulation of connective tissue that can ultimately have severe pathophysiological effects. The consequences of this pathophysiology derived from chronic muscle injury are again apparent in *mdx* dystrophy. Following the acute onset of inflammation of *mdx* muscle, which is dominated by a Th1 inflammatory response, *mdx* muscle regenerates and then begins a continuously progressive pathology that is dominated by a Th2 response in which M2a and M2c macrophages are the most prevalent leukocytes. During this progressive stage, fibrotic tissue accumulates continuously in muscle (182) contributing to loss of ambulation when the muscles of locomotion become fibrotic and causing severely impaired respiratory capacity, as the diaphragm becomes fibrotic (221). However, despite the continuous presence of M2a macrophages throughout the progressive, fibrotic stages of muscular dystrophy and the potent, profibrotic activity of TGFβ that can be produced at high levels by M2 macrophages, TGFβ appears to contribute to the fibrotic process only during early stages of the dystrophin pathology. For example, TGFβ expression in DMD muscle is elevated early in the disease, but its expression then declines while fibrosis continues to progress (16). Muscles of *mdx* mice muscles also show elevations in TGFβ mRNA at early stages of the disease but the levels then decline so that they do not differ significantly form wild-type muscles as the fibrosis continues (257). Likewise, dystrophin-deficient dogs show a reduction in TGFβ expression even while fibrosis progresses (181). Experimental findings also indicate that TGFβ is most important early in the *mdx* pathology. Depletions from *mdx* mice of T-lymphocytes that have the capacity to regulate the Th1 versus Th2 balance *in vivo* produced large reductions in circulating TGFβ levels, but the depletions had no effect on muscle fibrosis (160).

The later, progressive fibrosis of *mdx* muscle appears to occur through TGFβ-independent mechanisms that nevertheless involves M2 macrophages. In particular, the elevated expression of arginase in M2 macrophages in *mdx* muscle may be sufficient to drive the slow, progressive fibrosis in these chronically injured muscles. Hydrolysis of arginine by arginase leads to the production of ornithine that is subsequently metabolized for the production of proline (Fig. 15) (278). Proline generated through this pathway then contributes to the pool necessary for production of the proline- and hydroxyproline-rich connective tissue proteins in the collagen family (13, 48). During embryonic development or during wound healing following injury, connective tissue production can be limited by arginine availability for arginase (13, 276, 277). Furthermore, the substrate limitation of arginine for arginase-mediated production of connective tissue is influenced by substrate competition with NOS for their common substrate (12). However, during *mdx* dystrophy and DMD dystrophy there is a pathological reduction of nNOS in muscle fibers (25) and this nNOS deficiency leads to increased availability of arginine for arginase. This increased arginase availability combined with the chronic and long-term course of muscle injury and repair contributes significantly to the pathological fibrosis of dystrophic muscles (271).

Does depletion of the regenerative capacity of satellite cells play a role in the pathology of chronic muscle injuries?

Satellite cells, like most other diploid cells, are able to undergo a limited number of cell divisions *in vitro*, which presumably reflects a limit to their replicative ability *in vivo*. Similar to other cells that experience replicative senescence, satellite cells from rodents and humans typically experience 40 to 70 doublings of their population *in vitro* before their ability to enter the cell cycle is exhausted (50, 203, 213). This is a surprisingly low number of replications for a tissue in which satellite cells can be routinely activated as a consequence of exercise, as well as injury. Furthermore, a limit on the replicative capacity of satellite cells suggests the possibility that the ability of muscle to repair and regenerate may be

limited by the number of cycles of division experienced by a satellite cell and its progeny.

Because dystrophic muscles experience chronic injury that produces repeated rounds of satellite cell activation, they provide a valuable model for testing the expectation that the replicative senescence of satellite cells can limit repair and regeneration of skeletal muscle. *In vitro* studies strongly support that expectation. For example, the number of rounds of division that single cells that were isolated from muscle would undergo in culture was smaller if the cells were isolated from the muscle of DMD patients than if they were obtained from the muscle of healthy age-matched controls (19). These defects in regenerative capacity of DMD muscle cells *in vitro* are severe early in the disease; although muscle cells from a 5-year-old, healthy boy completed 56 rounds of division *in vitro*, muscle cells from 7-year-old DMD boys could seldom complete 10 rounds (269). Thus, these findings suggest that exhaustion of the replicative ability of satellite cells in chronically injured muscle could contribute to impairments in muscle regeneration. However, *in vivo* data do not provide definitive support for that interpretation. For example, electron microscopic observations showed that the number of satellite cells per muscle fiber in DMD patients in whom muscle regeneration was apparently impaired significantly exceeded the number of satellite cells in control adults or children (101). Likewise, data collected on isolated muscle fibers from *mdx* mice do not agree well with the expectation that a loss of satellite cell proliferative capacity contributes substantially the loss of the regenerative capacity of old *mdx* muscle. Muscle fibers from old *mdx* muscles in which the disease is progressive and resembles the pathology of DMD, showed significantly more satellite cells on their surface than were present on wild-type fibers (20). Furthermore, the number of satellite cells on the surface of muscle fibers of old *mdx* fibers exceeded the number present on young *mdx* fibers (20), contrary to the expectation that the replicative capacity of satellite cells was depleted as the disease progressed.

The differences in the apparent, relative, replicative capacity of dystrophic satellite cells assayed *in vivo* versus *in vitro* may reflect differences in their response to their environments. Increased oxidative stress may be the most important among the many nonphysiological stresses applied to satellite cells *in vitro* that may have differential effects on dystrophic and wild-type satellite cells. Typical, *in vitro,* cell culture conditions place cells in 5% carbon dioxide and ambient pO_2, which is approximately 20% oxygen. *In vivo*, muscle cells exist at approximately 2% to 6% O_2 (62, 95, 119). This difference in pO_2 can have tremendous effects on proliferation of satellite cells and on their patterns of gene expression. For example, satellite cells grown *in vitro* at 6% O_2 are three times more proliferative than those grown in 20% O_2 (46) and single satellite cells at 6% O_2 express Myf5 at 2-fold higher and MyoD at nearly 0.5-fold higher levels than expressed at 20% O_2 (46). The effects of increased oxidative stress may be particularly important for satellite cells isolated from *mdx* or DMD muscles. Dystrophin-deficient myotubes are more susceptible to lysis by oxidative stress than wild-type myotubes (56). However, dystrophin-deficient muscle cells are not more susceptible to killing by other metabolic stresses, which indicates that their increased frailty is a more specific susceptibility to oxidative stress (56). Thus, the reduced replicative capacity of *mdx* muscle cells *in vitro* could reflect reduced viability induced by oxidative stress rather than loss of regenerative capacity caused by more numerous rounds of activation in chronically injured muscle.

Conclusion

Although skeletal muscle injuries can result from a wide range of mechanical, environmental, or genetic perturbations, several common features are associated with initiating muscle injury and the processes of repair and regeneration that follow. With the exception of direct mechanical damage to myofibrils that occurs during eccentric contractions, lesions of the muscle cell membrane during acute injuries appear to be a key event in propagating muscle injury and initiating repair and regeneration. Many of the events that follow muscle membrane damage that affect the course of injury, repair, and regeneration are attributable, at least in part, to the influx of calcium from the extracellular space into the injured muscle cell. On one hand, unregulated calcium influx can exacerbate damage. Calpain activation by elevated calcium can cause proteolysis of many essential structural and regulatory proteins in the injured muscle, further worsening damage. Supraphysiological activation of PLA_2 can cause further damage by causing excessive hydrolysis of membrane phospholipids, leading to further membrane defects. In addition, metabolism of the hydrolysates that are derived from PLA_2 activity yields chemoattractants for inflammatory cells that can cause further muscle damage via the production of cytolytic free radicals.

On the other hand, calcium influx can also play a central role in initiating muscle repair and regeneration. For example, increased calcium elevates NO production by nNOS which in turn increases the activation of proteases that release FGF2 and HGF from their inactive state, bound to the extracellular matrix. Release of HGF and FGF2 causes satellite cell activation and stimulates satellite cell proliferation and migration to injury sites, thereby increasing muscle repair and regeneration. Furthermore, myeloid cell invasion that may be initiated, in part, by calcium activation of PLA_2, can contribute to muscle repair and regeneration. Although the early invading population of neutrophils and M1 macrophages contribute to muscle cell lysis, they also generate Th1 cytokines that can increase satellite cell proliferation and chemotaxis. The M1 macrophages can then be deactivated and switch to a M2 phenotype that promotes differentiation, regeneration, and growth of the injured muscle. Elevations in cytosolic calcium also increase membrane repair by enabling dysferlin and annexins to bind one another and to bind membrane phospholipids as part of the membrane-resealing process. Thus, membrane lesions can allow the influx of calcium that initially promotes

further membrane damage but can also contribute to its eventual repair.

Many basic and applied questions concerning the injury, repair, and regeneration of skeletal muscle remain. The incomplete understanding of the specific mechanical parameters that cause muscle damage during eccentric contractions is surprising. For example, why does the relative importance of strain, strain rate, force, and work in the occurrence of muscle injury during eccentric contractions differ between investigations? Do the differences reflect structural differences in the muscles studied, the metric used to assess injury, the conditions used to induce injury or some other variable? Can a better understanding of the specific mechanical parameters that cause myofibril damage aid in the design of exercise or therapeutic protocols that minimize muscle injury?

Improved knowledge of the biology of satellite cells *in vivo* would greatly advance understanding of muscle injury, repair, and regeneration. The uncertainty of whether replicative senescence of satellite cells that has been described *in vitro* is important in influencing the capacity of muscle to repair or regenerate *in vivo* is a major and surprising gap in current knowledge. Furthermore, the extent to which the renewal capacity of satellite cells is influenced by exogenous factors *in vivo* has been little explored. Given that satellite cells can follow their myogenic program *in vitro* in the absence of other cell types, does the experimental manipulation of exogenous factors *in vivo* such as growth factors, cytokines, or chemokines affect the final extent of muscle regeneration following injury, or only affect the rate of regeneration? Furthermore, little is known of the complex interactions that may occur in injured muscle *in vivo* between the oxidative stress that commonly accompanies intense muscle use or muscle injury and the changes in patterns of gene expression in satellite cells.

Broader knowledge of the effects of *in vivo* manipulations of cytokines that influence muscle injury, growth, and regeneration is needed. Gaps in our knowledge of cytokine-mediated mechanisms that affect muscle injury and regeneration include an understanding of how the multiple signaling pathways that are activated in inflamed muscle interact. Our current understanding of the influence of cytokine-induced signaling in muscle is founded primarily on studies conducted *in vitro*, in which the effects of a single cytokine on muscle are tested. While those *in vitro* studies are essential first steps in understanding the potential influences of cytokines on muscle repair and regeneration following injury, they may not be truly predictive of the roles played by selected cytokines in the more complex, *in vivo* environment in which the expression of scores of regulatory proteins and their receptors can rapidly fluctuate.

Finally, more specific information is needed concerning the regulatory roles played by specific inflammatory cell populations in the process of muscle injury, repair, and regeneration. For example, is it possible to distinguish between macrophage subpopulations that drive fibrosis and those that promote repair and growth? Is there a phenotypic distinction between the M2 macrophages that are involved in chronic muscle injuries and those that promote repair and regeneration following acute injury? Is it possible to manipulate specific subpopulations of myeloid cells *in vivo* so that their contributions to muscle injury are minimized and the effects on repair and regeneration are amplified? Although answering these questions is important for advancing our understanding of muscle injury, repair, and regeneration, the knowledge gained will also be pertinent to advancing our understanding of injury and repair mechanisms in other tissues. Once again, skeletal muscle provides an ideal system for studying central issues in physiology.

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