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# Immunobiology of Inherited Muscular Dystrophies

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## Abstract

The immune response to acute muscle damage is important for normal repair. However, in chronic diseases such as many muscular dystrophies, the immune response can amplify pathology and play a major role in determining disease severity. Muscular dystrophies are inheritable diseases that vary tremendously in severity, but share the progressive loss of muscle mass and function that can be debilitating and lethal. Mutations in diverse genes cause muscular dystrophy, including genes that encode proteins that maintain membrane strength, participate in membrane repair, or are components of the extracellular matrix or the nuclear envelope. In this article, we explore the hypothesis that an important feature of many muscular dystrophies is an immune response adapted to acute, infrequent muscle damage that is misapplied in the context of chronic injury. We discuss the involvement of the immune system in the most common muscular dystrophy, Duchenne muscular dystrophy, and show that the immune system influences muscle death and fibrosis as disease progresses. We then present information on immune cell function in other muscular dystrophies and show that for many muscular dystrophies, release of cytosolic proteins into the extracellular space may provide an initial signal, leading to an immune response that is typically dominated by macrophages, neutrophils, helper T-lymphocytes, and cytotoxic T-lymphocytes. Although those features are similar in many muscular dystrophies, each muscular dystrophy shows distinguishing features in the magnitude and type of inflammatory response. These differences indicate that there are disease-specific immunomodulatory molecules that determine response to muscle cell damage caused by diverse genetic mutations.

## Introduction

Skeletal muscle damage is a routine event that occurs throughout life, as a consequence of acute trauma, perturbations of blood supply, or increased muscle use. Even when damage is minor, it initiates a response in which complex and coordinated interactions between muscle and the immune system influence the course of muscle repair, regeneration, and growth. Just as in other tissues, the initial immune response to muscle damage consists of an ancient form

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of immunity, called innate immunity, in which phagocytic, cytolytic, and secretory inflammatory cells are rapidly mobilized and activated to enter the damaged tissue where they remove debris and promote repair. Although the innate immune response following acute injury is attributable to natural selection for processes that identify, kill, and remove invading infectious organisms, such as bacteria and parasites, the innate immune system is also activated in sterile injuries by endogenous molecules that are released by damaged tissue. As a result, innate immunological mechanisms that may have developed initially as evolutionary adaptations to acute, infectious events can also participate in muscle repair following acute, sterile injuries.

The relatively frequent and potentially lethal occurrence of acute injuries that are infected provide strong selective pressure for an innate immune response that is rapidly responsive to acute damage. However, chronic injuries also cause an inflammatory response that is dominated by innate immunity, although the relatively rare occurrence of chronic damage provides less selective pressure for immunological responses that are more specifically adapted to respond to chronic rather than acute injuries. Thus, inflammatory mechanisms that may be primarily adaptive to injury and repair processes that are normally resolved in days can persist in chronically injured tissues for the entire life of the organism.

The muscular dystrophies are the most common of the chronic muscle diseases that are associated with an innate immune response. Although the muscular dystrophies constitute a group of more than 50 genetically distinct diseases (168), they are grouped into a single, disease superfamily that is characterized by progressive muscle weakness and degeneration. The most frequently occurring muscular dystrophies also involve damage to the muscle cell membrane, which can lead to profound disruption of homeostasis, as well as chronic inflammation and fibrosis that are secondary, downstream consequences of the molecular defects causing the diseases. As shown by a growing body of evidence, many aspects of the innate immune response to chronic muscle injury that occurs in some muscular dystrophies are maladaptive and can contribute to amplifying rather than resolving the pathology.

Our goal in this review is to present current knowledge of regulatory interactions between muscle tissue and the immune system in muscular dystrophies. Mechanisms through which inflammation of dystrophic muscle can either worsen pathology or improve regeneration are examined, exploring the hypothesis that detrimental interactions between the immune system and dystrophic muscle are attributable, in part, to an innate immune response adapted to acute tissue injuries that is operating in a chronically injured and inflamed tissue. In addition, we present evidence that perturbations in the expression or activity of endogenous immunomodulators can influence interactions between muscle and the immune system that are specific to different muscular dystrophies. Finally, discoveries are presented which show that the immune response to dystrophic muscle extends beyond innate immunity in which myeloid cells are the primary effector population, to include components of the acquired immune system, in which the actions of lymphoid cells are of central importance. Although muscular dystrophies include a large number of distinct diseases, the majority of this review concerns the immunobiology of muscular dystrophies that result from mutations of genes that encode the proteins dystrophin or dysferlin because our current understanding of immune cell involvement in those muscular dystrophies is the most developed. However, we

also present the relatively limited information concerning the possible involvement of the immune system in the pathophysiology of other muscular dystrophies, to emphasize similarities and differences in the immune response in those diseases and to highlight areas in which further research is needed.

## The Immunobiology of Duchenne Muscular Dystrophy

### Overview of Duchenne muscular dystrophy

Duchenne muscular dystrophy (DMD), the most common muscular dystrophy, is one of the most frequent, lethal, genetic diseases of childhood (Fig. 1). Because DMD is an X-linked recessive disease, it almost exclusively afflicts males. DMD and a milder form of muscular dystrophy, Becker muscular dystrophy (BMD), are caused by mutation of the gene that encodes a membrane-associated structural protein, called dystrophin (148). Collectively, DMD, BMD, and animal models in which dystrophin is deficient are “dystrophinopathies” that share many common pathological features but may vary in severity and disease progression.

Much of the pathology of dystrophinopathies is attributable to the loss of mechanical functions served by dystrophin. Dystrophin normally binds to the actin cytoskeleton and links the cytoskeleton to a transmembrane complex of proteins, the dystrophin-associated glycoprotein complex (DGC) (87) (Fig. 2). The extracellular domain of the DGC binds to laminin, an extracellular matrix (ECM) protein, thereby forming a chain of molecules that link intracellular and extracellular structural proteins (157). Soon after the discovery of dystrophin, the protein was expected to function in providing mechanical support to the muscle cell membrane because of its location, its binding partners, and its domains that share structural similarities to known cytoskeletal proteins, spectrin and α-actinin (173). Subsequent experimentation validated that membranes of dystrophin-deficient muscles were mechanically weaker than membranes of healthy muscle fibers (259), which could be demonstrated by increased efflux from dystrophic muscle of cytosolic proteins, such as muscle creatine kinase (CK), and increased influx of extracellular marker dyes, such as Evans blue (205) (Fig. 3). These findings established the “mechanical defect hypothesis” as a basic canon for the pathogenesis of DMD, stating that the absence of dystrophin produced a weaker cell membrane that tore more easily, leading to unregulated influx/efflux of molecules and severe disruption of homeostasis and cell death.

### Defects in the mechanical defect hypothesis

Although the mechanical defect hypothesis explains some major features of the pathophysiology of DMD and the pathology that occurs in the *mdx* mouse, a genetic model of DMD, many features of the natural history of the disease cannot be simply explained by the hypothesis. For example, dystrophin-deficient muscles of mice and men experience increased fatigability, fibrosis, and fat deposition and defects in regeneration, vasoregulation, metabolism, and synaptic structure and function (63, 75, 81, 98, 202, 234, 279, 280, 289, 315, 336, 350, 356). DMD boys and *mdx* mice both experience cognitive defects, that appear to be partly attributable to impaired consolidation of short-term memories (51, 142, 143). In addition, the clinical onset of the disease in DMD boys occurs at about 3 years of

age (244) and *mdx* mice display no histopathology until the acute onset of pathology at about 3 to 4 weeks of age, although they are physically active and do not express dystrophin during the period that precedes the onset of pathology. Furthermore, although the loss of dystrophin causes a continuously progressive, lethal disease in humans and in the canine model of DMD (GRMD dogs), the same molecular defect in mice permits a period of successful regeneration of muscle that begins at about 2 months of age and persists to about 12 months of age, after which the pathology is continuously progressive. Collectively, the diverse and complex pathology of dystrophin deficiency indicates that disruption of multiple effector systems are secondary consequences of dystrophin deficiency that contribute significantly to the disease.

## Macrophages are primary effectors of muscle membrane damage in *mdx* dystrophy

Inflammation is a characteristic feature of dystrophin-deficient muscle, although whether inflammatory cells contribute significantly to the pathology of DMD or *mdx* dystrophy was debated for decades. On one hand, administration of the immunosuppressing corticosteroid, prednisone, to DMD patients can reduce and slow progression of DMD pathology. Prednisone-treated DMD patients can maintain muscle strength and function, prolong ambulation, and show reduction in secondary musculoskeletal defects such as scoliosis, compared to nontreated DMD patients (22, 33, 92, 281, 371). Prednisone treatments also retain respiratory function in *mdx* mice (123), which is a particularly important outcome because respiratory dysfunction that is secondary to the loss of dystrophin is a primary cause of death in DMD patients. In addition, prednisone treatments significantly reduced the numbers of inflammatory cells in dystrophin-deficient muscle (171, 354). Together, the observations supported the view that inflammation promotes the pathology of dystrophin deficiency and that prednisone's beneficial effects are mediated through its functions as an immunosuppressant. On the other hand, treatment of DMD patients with another immunosuppressant, azathioprine, did not yield clinical improvements similar to prednisone, suggesting the possibility that prednisone did not ameliorate DMD by acting as an antiinflammatory (127). However, azathioprine is a purine analog that inhibits DNA synthesis in all cells, not only inflammatory cells. Thus, azathioprine treatments could inhibit the expansion of muscle progenitor cell populations that are necessary for muscle repair and regeneration, which could contribute to a lack of improved muscle function in azathioprine-treated DMD patients.

Despite the strong evidence that prednisone functions as an antiinflammatory agent in dystrophic muscle and that prednisone treatments yield beneficial effects on function in DMD patients and *mdx* mice, whether the benefits are only attributable to its immunosuppressive effects remains uncertain because prednisone has other, nonimmunosuppressive effects. For example, prednisone can affect myogenesis (254), reduce proteolysis (15, 271), affect metabolism (271), reduce myonuclear apoptosis (295), and increase expression of the dystrophin homolog, utrophin (64). Any of those effects could reduce the pathology of dystrophin deficiency, independent of effects on inflammatory cells. Nevertheless, more direct manipulations of inflammatory cell populations provide clear evidence that they are responsible for a major portion of damage to dystrophin-deficient muscles, at least in *mdx* muscular dystrophy. In particular, macrophage populations can be

depleted by intraperitoneal injections of antibodies to a monocyte/macrophage cell surface protein (F4/80) that bind the antigen. The targeted cell type is then coated, or opsonized, with antibody which can lead to its selective removal by other phagocytes or by activation of the complement system (3) (Fig. 4A). Whether this depletion only affects macrophages that are derived from circulating leukocyte populations or also macrophages that reside in healthy muscle is unknown. This question may be relevant to understanding the pathogenesis of muscular dystrophy because circulating and resident macrophage populations can have different developmental origins and may serve different functions in injured or diseased tissues (187). Performing these antibody depletions of macrophages before the acute onset of pathology in *mdx* mice produced nearly an 80% reduction in the number of muscle fibers with a detectible, unregulated influx of marker dye into the cytosol at the acute peak of pathology (351), showing that a large proportion of dystrophic muscle membrane damage is a consequence of macrophage-mediated membrane events, not mechanical damage (Fig. 4B, C, D). Collectively, these observations suggest that the pathology of dystrophic muscle during early childhood in humans or early postnatal growth in mice may be subclinical because those periods precede the onset of muscle inflammation. The subsequent inflammation then amplifies muscle membrane damage by 400% to 500%, and may be necessary for the clinical onset of the disease.

## Macrophages cause muscle membrane lesions through the release of cytolytic free radicals

Macrophages and other myeloid cells that most rapidly invade injured tissue can generate free radicals at high concentrations that are sufficient to cause cell lysis. This system is highly adaptive for killing infectious organisms that may invade tissue following acute injuries. It is also adaptive for facilitating tissue repair following acute injuries because oxidation of debris produced by acute injury targets the debris for phagocytosis and clearance by macrophages and other phagocytes. However, the nonspecificity of the free radical-mediated cytolysis also creates a risk for myeloid cell-mediated damage of healthy, host tissues and that risk is even greater in chronic injuries. Thus, the rapid invasion of dystrophic muscle by macrophages at the clinical onset of the disease may be an adaptive response to acute muscle injury that can actually amplify tissue damage when the injuries are chronic.

During the initial inflammatory response to muscular dystrophy in *mdx* mice, macrophage populations are dominated by a phenotype that is specialized for free-radical production and phagocytosis. Although macrophage phenotypes are distributed along a broad spectrum in which their patterns of gene expression and functions are influenced by the microenvironment in which they are activated, the ends of the spectrum are represented by M1 macrophages and M2 macrophages (Fig. 5). M1-biased macrophages are activated to a phenotype that is specialized for free-radical production by proinflammatory cytokines that induce expression of inducible nitric oxide synthase (iNOS). Tumor necrosis factor (TNF) is particularly important in inducing iNOS expression in macrophages, neutrophils, and other cell types by activating the transcription factor NFκB, which increases the expression of iNOS and other proinflammatory mediators that further drive macrophages toward the M1 phenotype. The primary function of iNOS is to metabolize arginine to generate the free-

radical nitric oxide (NO) and citrulline. However, unlike other isoforms of NOS that generate low levels of NO that function in cell signaling and are constitutively expressed, the high levels of NO production by iNOS can drastically change the redox environment in injured tissue and react with other free radicals to amplify their reactivity and toxicity. Lysis of the cell membranes of infectious organisms or by-standing host cells is a primary consequence of elevated NO production by iNOS, and *in vitro* and *in vivo* data show that much of macrophage-mediated muscle membrane lysis is attributable to iNOS. For example, muscle cell lysis by *mdx* muscle-derived macrophages *in vitro* is prevented by iNOS inhibition (342). Also, null mutation of iNOS in *mdx* mice caused significant reductions of muscle membrane lysis at the acute onset of pathology (342). Nevertheless, iNOS ablation in a dystrophin-deficient mouse model with a more severe phenotype ($mdx^{4cv}$) did not cause improvements in muscle contractility (191), showing that some important features of the pathology of dystrophinopathies are independent of iNOS-derived NO. In addition, the reduction of muscle membrane damage caused by iNOS ablation in *mdx* mice was less than that achieved in macrophage-depleted *mdx* muscle (351), indicating that macrophages also increase *mdx* pathology through iNOS-independent mechanisms that have not yet been identified.

The significant contribution of macrophage-derived NO to muscle membrane damage in *mdx* muscular dystrophy indicates that mechanisms that influence the expression or activity of iNOS could significantly affect the pathology of *mdx* dystrophy. Much of this potentially beneficial, endogenous regulation may be achieved by a population of macrophages that are biased toward an antiinflammatory, M2-phenotype (Fig. 5). Following muscle damage, proinflammatory M1-biased macrophages that express iNOS (iNOS+) are replaced by iNOS-negative (iNOS-) macrophages that express CD163 and are biased toward the M2 phenotype (342). CD163 is a transmembrane glycoprotein in macrophages (283) for which expression is strongly influenced by cytokines. In particular, proinflammatory TNF suppresses CD163 expression but the antiinflammatory cytokine, interleukin-10 (IL-10), induces CD163 expression (41, 283, 305). In turn, ligation of CD163 by hemoglobin-haptoglobin complexes that are elevated in injured tissue increases IL-10 expression (260), creating a positive feedback system, which can further drive macrophages toward an M2-biased population that supports muscle regeneration and repair following acute injury or disease.

The elevated levels of IL-10 that result from the feedback system can deactivate the M1 phenotype and reduce expression of iNOS by inhibiting NFκB signaling. The regulatory importance of IL-10-mediated signaling in dystrophic muscle was demonstrated in *mdx* mice in which IL-10 expression was ablated. Macrophages from IL-10 null/*mdx* muscles were more cytolytic *in vitro* than macrophages from IL-10-expressing *mdx* muscles and muscle damage was more extensive in IL-10 null/*mdx* mice *in vivo* (343). In addition, IL-10 ablation reduced the numbers of M2-biased macrophages in *mdx* muscles and decreased muscle function during regenerative stages of *mdx* muscular dystrophy (343).

M2-biased macrophages can also reduce iNOS activity, in addition to reducing expression of iNOS. A subpopulation of M2-biased macrophages, called M2a macrophages in some nomenclatures, express arginase. This is significant in the context of tissue injury and repair

because arginine availability in injured or developing tissues can limit the activity of both iNOS and arginase, both of which require arginine as substrate. Typically following acute injuries most arginine in injured tissue is converted to citrulline during the first 2 days postinjury, reflecting iNOS metabolism of arginine to citrulline and NO by M1-biased macrophages (288). However, during the repair and regeneration stage that occurs 3 to 15 days postinjury, most arginine is metabolized to ornithine and urea by arginase, which reflects the phenotypic conversion of macrophages to M2-biased, arginase-expressing cells (288). In *mdx* muscles, the chronic elevation of both M1-biased and M2-biased macrophages creates a scenario in which the cytolytic capacity of iNOS-expressing macrophages may be influenced by the relative levels of expression and activity of iNOS and arginase in macrophages (Fig. 6). The influence of this substrate competition between macrophages on muscle cell lysis has been demonstrated *in vitro* where iNOS-mediated lysis of muscle cells in cocultures with interferon-gamma (IFNγ)-stimulated M1-biased muscle macrophages is significantly diminished by adding IL-4-stimulated, M2-biased, muscle macrophages to the cocultures (342). Thus, arginase can function as an endogenous immunomodulatory enzyme by functioning as a negative regulator of macrophage-mediated cytotoxicity.

Therapeutic or dietary interventions that reduce the expression of iNOS by macrophages and other cells in *mdx* muscle also reduce pathology and improve function, although those interventions do not specifically target iNOS. For example, treatment of *mdx* mice with curcumin, a potent inhibitor of NFκB, caused reductions in iNOS expression and decreased muscle membrane damage, and improved muscle function (250). In addition, using genetic or pharmacological interventions to specifically target NFκB signaling or to impede mechanisms that activate NFκB in dystrophic mice also produced reductions in muscle membrane lysis and large improvements in muscle morphology and function (2,192). However, whether those beneficial effects were attributable to reductions in iNOS expression was not tested.

## The myeloid cell infiltrate at the early onset of pathology in dystrophin-deficient muscle is complex

**Neutrophils—**Although macrophages are the most prevalent inflammatory cell type at early stages of dystrophinopathy in *mdx* mice, when they can reach concentrations that can exceed $7 \times 10^4$cells/mm$^3$ of muscle (351), other myeloid cells also invade and influence the dystrophic pathology. Neutrophils are particularly prominent among the early invading population when they are approximately 30% as numerous as macrophages in *mdx* muscles (342). They also play a significant role in promoting muscle damage at this stage of pathology. Antibody depletions of circulating neutrophil populations before the acute onset of *mdx* pathology produced a reduction in histologically discernible muscle damage and inflammation at the acute peak of pathology by over 80% (147). Neutrophils may also impair regeneration of dystrophic muscle. Neutrophil elastase, a protease released by neutrophils and macrophages during inflammation, reduces viability of muscle cells *in vitro* and impairs their differentiation (12). Although neutrophil involvement in muscle damage has not been tested in human, DMD muscle, they may possibly play an even more injurious role in DMD than *mdx* muscular dystrophy; neutrophils comprise approximately 60% of

circulating leukocyte populations in humans, but are only 10% to 25% of circulating leukocytes in mice (213).

The tremendous reductions in *mdx* muscle damage that are achieved by depleting either neutrophils or macrophages from *mdx* mice before the acute onset of pathology show that both populations are attractive targets for therapeutic interventions. In addition, the similarly large reductions in muscle damage are achieved by depletion of either neutrophils or macrophages (~80%) indicate that the two myeloid populations likely act primarily through a common cytolytic mechanism (Fig. 7). Although this possibility has not been explored explicitly in dystrophic muscle *in vivo*, *in vitro* observations indicate mechanisms through which cooperative interactions between neutrophils and macrophages can promote muscle cell lysis. For example, the presence of small numbers of neutrophils in cocultures of muscle cells and macrophages amplified macrophage killing of muscle cells by NO-dependent mechanisms (242), possibly attributable to highly cytolytic free radicals produced by reactions between macrophage- and neutrophil-derived molecules. In one possible scenario, the reaction of neutrophil-derived superoxide with macrophage-derived NO would produce peroxynitrite, a highly reactive free radical that could amplify muscle damage. However, whether peroxynitrite levels are significantly elevated in *mdx* muscle is disputed (78, 151, 320).

Whether neutrophils can independently and directly cause lysis to dystrophic muscle fibers has not yet been definitively demonstrated. However, they can directly and independently lyse nondystrophic muscle cells *in vitro* via free-radical-mediated mechanisms (241) and dystrophic muscle cells are more sensitive to damage by free radicals (78). Much of their muscle lytic activity *in vitro* results from myeloperoxidase (MPO)-mediated processes (241). MPO is expressed primarily by neutrophils which can secrete it at sites of inflammation, where MPO can oxidize chlorides in the presence of hydrogen peroxide to generate hypochlorous acid, which is a highly reactive oxidant capable of causing tissue damage. MPO activity in *mdx* muscles is approximately seven-times greater than in wild-type muscles and MPO in muscles of dystrophin-mutant dogs (GRMD dogs) is more than 30-times higher than in wild-type dog muscles (312, 313). In addition, the magnitude of increase in neutrophil numbers in GRMD muscles is similar to the increase in MPO (312), supporting the interpretation that neutrophils are the primary source of MPO in dystrophic muscles. However, whether neutrophil MPO promotes oxidative damage to dystrophic muscle is uncertain. On one hand, treating *mdx* mice with taurine reduced muscle MPO and reduced protein thiol oxidation (313); taurine is a conditional amino acid that is important in fat metabolism and can also function as an antioxidant. Those observations support the interpretation that neutrophil-derived MPO increases damage of dystrophic muscle via increased oxidative damage. However, treating *mdx* mice with L-2-oxothiazolidine-4-carboxylate (OTC) produced similar reductions in muscle MPO, but did not affect protein thiol oxidation. OTC is a cysteine precursor that is antioxidative, in part, by increasing glutathione production. Those findings suggest that increases in muscle oxidative damage in *mdx* mice are not affected by MPO activity. Alternatively, MPO may cause oxidative damage that is undetectable in assays of protein thiol oxidation.

**Mast cells—**Mast cells are a population of myeloid cells that play key roles in the innate immune response and help serve as an immunological barrier at sites where antigens or infectious organisms can enter the body. However, they also serve more nuanced roles through the release of chemokines and cytokines that can amplify the inflammatory response. For example, they are rich sources of the proinflammatory cytokine TNF (26, 117), but they also release potent, antiinflammatory cytokines, such as IL-10 (103, 128, 214). Mast cells also release vasoactive substances such as histamine that can influence peripheral blood flow and proangiogenic factors, such as vascular endothelial growth factor (VEGF). Similar to macrophages and neutrophils, they produce free radicals that can kill infectious organisms but can also damage bystander, host cells. They can also release cytokines such as transforming growth factor-beta (TGFβ) that promote connective tissue accumulation, but also proteases such as chymases and tryptases that increase connective tissue breakdown (47). Mast cells share with other myeloid cells the characteristic that they appear to have acquired their functional specializations under selective pressures to rapidly respond and protect from pathogens and infectious organisms that occur acutely and commonly. However, also similar to other myeloid cells, chronic elevations in mast cell numbers or activity can increase pathology. For example, the presence of large numbers of activated mast cells contributes to asthma, and their prolonged presence is associated with atherosclerosis and even aortic aneurysms (13, 56, 306).

The apparently antithetical functions of mast cells in injured and diseased tissue are also apparent in DMD and *mdx* dystrophic muscles. Early histological observations showed that mast cells are present in normal, healthy human and rodent muscles, but their numbers were tripled in DMD muscle (139) and elevated six- to ten-fold in *mdx* muscles (189, 235). However, their locations in dystrophic muscle indicated more than one role for them in the pathology of DMD; elevated numbers were observed in regions in which necrotic muscle fibers dominated, but also at sites of regeneration and fibrosis, suggesting potential mast cell involvement in both damage and repair (139). In an attempt to resolve the primary function of mast cells in injured muscle, the same investigators examined the time course of mast cell accumulation in acutely injured muscle and observed that the greatest increase in mast cell numbers occurred during the regenerative stage following muscle damage, implicating mast cells in a proregenerative role. Subsequent experimental observations also emphasized the relationship between the numbers and location of mast cells and the regeneration of *mdx* muscle (189). However, the relationship may vary between muscles. For example, in *mdx* soleus and gastrocnemius muscles there is a continuous increase in mast cell numbers between the early acute peak of *mdx* pathology (4–5 weeks of age) and 1 year of age, suggesting mast cell involvement in regeneration and fibrosis (189, 275). However, mast cell numbers in the tibialis anterior (TA) declined continuously over the same period (189).

Despite the potentially opposing roles of mast cells in dystrophic muscle, experimental findings showed that the net effect of the acute release of mast cell granules in dystrophic muscle was detrimental, at least at some stages of the disease in *mdx* mice. Intramuscular injection of isolated mast cell secretory granules, which would contain a complex mix of secretory molecules including histamine, proteases and cytokines, caused a significant increase in *mdx* muscle damage (121), suggesting that inhibition of mast cell degranulation

could reduce *mdx* pathology. This possibility was tested in *mdx* mice that received daily treatments up to 4 weeks of age with cromolyn, which reduced mast cell degranulation (264). Although cromolyn treatments caused a reduction in muscle damage in some muscles on some treatment days (e.g., in TA of 28-day-old mice) on other treatment days cromolyn administration was associated with elevated muscle damage (e.g., in TA of 24-day-old mice) (264). The possibility that inhibition of mast cell degranulation could reduce pathology in DMD has also been tested, using oxatomide which is an antihistamine that also inhibits mast cell degranulation (270). However, 6 months of oxatomide treatment of 5 to 10-year-old DMD patients produced no significant functional improvements (43). Although the function of mast cells in dystrophic muscle remains enigmatic, the negative or mixed findings in dystrophic mice and humans may not necessarily mean that mast cells are unimportant in regulating the response of muscle to dystrophin deficiency. Alternatively, the findings may reflect complex regulatory roles for mast cells in the disease, so that their disruption perturbs both injury and regenerative processes that are not separated by the intervention strategies tested to date.

**Eosinophils—**The initial invasion of injured, dystrophin-deficient muscle by macrophages, neutrophils, and mast cells resembles a nonspecific inflammatory response to injury that occurs in any tissue experiencing acute damage. However, deeper examination of leukocyte populations in dystrophic muscle at early stages of dystrophinopathy revealed unexpected immune cell populations, indicating that inflammatory mediators in muscular dystrophy differ from an expected, nonspecific inflammatory response. In particular, eosinophils are present at elevated numbers in *mdx* muscle at early stages of the disease, reaching peak concentrations of $4 \times 10^3$eosinophils/$mm^3$ of muscle (44) (Fig. 8). Eosinophils are typically constituents of innate type 2 immune responses that are driven by antiinflammatory cytokines (e.g., IL5) and are associated with allergic reactions or immune responses to infection by parasites (292). In responding to infections, eosinophils have the capacity to lyse parasites (268), with much of their cytolytic ability attributable to the release of granules of highly cationic proteins, such as major basic protein (MBP) (111) and eosinophil cationic protein (ECP) (277). However, eosinophils also have the ability to injure, neighboring by-stander cells; the release of either MBP or ECP from eosinophils is lytic to respiratory, neural, or cardiovascular cells (100, 112, 307) suggesting that eosinophils can worsen pathology in some diseases. Because corticosteroid treatments can greatly reduce eosinophil numbers and pathology in other inflammatory diseases (184), part of the beneficial effects of corticosteroids in DMD may result from reductions in eosinophil-mediated muscle damage.

Although eosinophils have potent, cytolytic functions, they also serve immunomodulatory roles in innate type 2 immunity, especially through their release of antiinflammatory, Th2 cytokines such as IL-4 (221, 236) and IL-10 (154, 236). In particular, their release of IL-10 can deactivate proinflammatory M1-biased macrophages, reducing macrophage expression of iNOS. Thus, the presence of eosinophils in dystrophic muscle could feasibly increase or decrease muscle membrane lysis. These functions were explored *in vivo* in *mdx* muscle by antibody depletions of eosinophils, using antibodies to the eosinophil cell-surface antigen called CC motif chemokine receptor-3 (CCR3), prior to the acute onset of pathology. Anti-

CCR3 treatments produced a 72% reduction in eosinophil numbers in *mdx* muscles at the acute onset of *mdx* pathology, which was associated with 67% reduction in the numbers of muscle fibers with detectible membrane lesions (357), showing that the net effect of eosinophils at this stage of *mdx* pathology is to promote membrane lysis. Although *in vitro* cytotoxicity assays showed that null mutation of MBP-1 in eosinophils greatly reduced their killing of muscle cells, *mdx* mice that were null mutants for MBP showed no reduction in muscle membrane lysis *in vivo*. Whether their *in vivo* cytolytic activities are attributable to release of other cationic proteins or occurs through less direct routes involving other cell populations is currently unknown.

## Lymphoid cells also promote muscle damage in dystrophinopathies

**Cytotoxic T-lymphocytes—**Although myeloid cells comprise the vast majority of leukocytes in dystrophin-deficient muscle, lymphoid cells are also present in the infiltrate where they can serve immunomodulatory and cytolytic roles. In particular, CD8+, cytotoxic T-lymphocytes (CTLs) occur in elevated numbers in DMD muscle (10, 84, 210), indicating that an acquired immune response may also affect the course of muscular dystrophy. In acquired immunity, CTLs have the ability to kill cells directly that are injured or infected and that express antigenic peptides on their surface in the context of major histocompatibility complex class 1 (MHC class 1) (Fig. 9). Early investigations into the inflammation of DMD and *mdx* muscle showed that there was an increase in the number of muscle fibers that expressed MHC class 1 and showed that all DMD fibers that were invaded by CTLs expressed MHC class 1 on their surfaces (84); this indicates that acquired immunity could be a feature of dystrophinopathy. Furthermore, analysis of the structure of the hypervariable domain of the T-cell receptor on CTLs in DMD muscle showed a highly conserved sequence in cells isolated from several DMD patients, indicating that CTLs were specifically activated by a common antigen in DMD muscle (131), also supporting an acquired component to the immune response to muscular dystrophy.

Several clinical observations are also consistent with a potential role for a CTL-mediated acquired immune response in DMD. For example, prednisone treatment of DMD patients under a treatment protocol that improved muscle strength and pulmonary function (36, 79, 92, 126, 171) was accompanied by a significant reduction of CD8+ CTLs in muscles (171). In addition, CD8+ T-cells and CD4+ T-cells in some DMD patients show dystrophin-specific immunity that is attenuated by prednisone (95). Furthermore, antibody depletions of CTLs from *mdx* mice produced significant reductions in muscle histopathology, specifically validating a role for CTLs in dystrophinopathies (297, 298). Similarly, mice that were dystrophin-deficient and were also mutants for $Prkdc^{scid}$ (*mdx/scid* mice) showed less fatigability in treadmill running and greater muscle force production than *mdx* mice (90). *Mdx/scid* mice are deficient in CTLs, which supports the possibility that the functional improvements in *mdx* mice resulted from CTL reductions. However, *mdx/scid* mice also lack other functional T-cell populations, such as CD4+ T-cells, which also influence muscle pathology in *mdx* mice (297).

The improvement in histopathology in CTL-depleted *mdx* mice could potentially result from reductions in direct, cytotoxic interactions between CTLs and MHC class 1 expressing

muscle fibers or it could be caused by disruption of other immunomodulatory roles played by CTLs. A potential, direct cytotoxic mechanism could result from engagement of T-cell receptors on the CTL surface with antigenic peptides presented with MHC class 1 on the diseased muscle fiber, causing the release of cytolytic proteins from the CTL. In particular, lysosomes within CTLs contain perforin, a cytolytic protein, and serine proteases that upon release can cause CTL-induced lysis of target cells (27). Genetic ablation of perforin in *mdx* mice caused reductions in muscle histopathology and produced a large reduction in the number of apoptotic muscle nuclei in muscle fibers (298). Because myonuclear apoptosis is an early feature of the pathology of dystrophinopathy, preceding the onset of muscle necrosis (317), CTL's influence on the disease may be particularly important at early stages.

**Interactions between cytotoxic T-cells and myeloid cells during early stages of muscular dystrophy—**CTLs are rich sources of cytokines that can influence the number and activation of other immune cell populations especially proinflammatory Th1 cytokines, such as TNF and IFNγ. Systemic deletion of IFNγ reduces *mdx* pathology, reduces inflammation, and shifts macrophages to an M2-biased phenotype that can increase muscle repair (343). However, whether CTLs are a primary cellular source of TNF and IFNγ in dystrophic muscle has not been established and myeloid cells that are present in much higher numbers can also release these cytokines. CTLs can also express chemokines such as CC-chemokine ligand 2 (CCL2) and CCL2 plays a central role in attracting myeloid cells into injured muscle (203,290). For example, in acutely injured, nondystrophic muscle, the genetic deletion of the CD8 alpha chain in CTLs reduced CCL2 production, causing reductions in macrophage recruitment following muscle injury (376). However, CTLs appear to play a less important role in recruiting macrophages to dystrophic muscle. Depleting CTLs produced only an insignificant trend for reductions in macrophage numbers in *mdx* muscles, although the depletions significantly reduced the invasion of individual dystrophic fibers by macrophages (298).

CTLs can also influence myeloid cell numbers and function through perforin-dependent cytotoxicity. Ablation of perforin expression in *mdx* mice caused reductions in muscle eosinophilia that were equivalent to reductions caused by prednisone treatments (44). Thus, part of the beneficial effect of CTL depletions or perforin mutations on *mdx* pathology may be attributable to reductions in muscle cytolysis by eosinophils. In return, eosinophils regulate CTL numbers in dystrophic muscle through an MBP-mediated pathway. Depletion of eosinophils or null mutation of MBP-1 in eosinophils produced large increases in CTL numbers in *mdx* muscles (357), showing that the CTL-driven pathology that is mediated by eosinophils can receive negative feedback from eosinophil-derived MBP-1 (Fig. 7). At least part of this negative feedback may reflect a direct immunomodulatory role played by MBP-1; treatment of splenocytes with MBP-1 *in vitro* significantly reduced their activation and proliferation (357).

## Activation of immune responses to muscular dystrophy

**Activation of the innate immune response—**Much of the complexity of the immune response to DMD or *mdx* dystrophy is attributable to two uncommon features of the diseases. First, the innate immune response to dystrophic muscle is essentially an acute

inflammatory response to tissue trauma that occurs chronically over the entire lifetime. Inflammatory mechanisms that would normally lead to tissue repair following a single injury instead lead to amplified damage and tissue fibrosis. In addition, dystrophic muscle simultaneously experiences an acquired immune response that not only increases muscle fiber death through direct cytolysis, it amplifies the innate immune response, which modulates and worsens pathology. Although activation of innate or acquired immune responses can occur independently, they are activated concurrently in dystrophic muscle, at least in *mdx* mice where time course data are available. That correspondence suggests there may be some shared regulatory mechanisms underlying their coactivation.

Rapid and robust activation of myeloid cells is an essential feature of innate immunity. In the context of infection, the speed of the response is vital because failure to identify and destroy infectious organisms can be lethal. The rapid response of myeloid cells to infectious organisms, such as bacteria, is possible because myeloid cells constitutively express receptors for specific molecular motifs, called pathogen-associated molecular patterns (PAMPs), that are within molecules on the surface of the infectious organism. Ligation of the PAMP receptors (pattern recognition receptors, PRRs) leads to myeloid cell activation, which initiates production of cytolytic molecules that can kill the infectious organism, induces phagocytosis, and causes the release of chemoattractant molecules to attract and activate other inflammatory cells (53). The endotoxin lipopolysaccharide (LPS) is a PAMP that binds to its receptor, toll-like receptor-4 (TLR4), that is expressed on myeloid cells and other cells to promote the inflammatory response. A similarly ancient system in innate immunity provides an equally rapid mechanism to respond to endogenous danger signals that are released in response to cellular damage, called damage associated molecular patterns (DAMPs). DAMPs may bind to their PRRs that are located either on the cell surface or within the cell, and their ligation can activate NFκB signaling to promote an innate immune response, elevate MHC class 1 expression and activate autophagy. For example, high mobility group box 1 protein (HMGB1) is a chromosomal protein that is normally sequestered in nuclei but can be released from injured cells into the extracellular space, where it functions as a particularly potent, extracellular DAMP. HMGB1 signaling through its cell surface receptor, TLR4, and the intracellular adapter protein MyD88 activates NFκB signaling, which can lead to increased production of proinflammatory cytokines and iNOS and promote inflammation (Fig. 10) (109, 253, 373). Single-stranded RNA (ssRNA) is a well-characterized intracellular DAMP that signals through the intracellular PRR TLR7 (138), which can also lead to activation of NFκB to drive the production of proinflammatory cytokines and iNOS (132, 324).

The importance of signaling through PRR in activating the immune response in dystrophinopathies is now well-established and TLR2 and TLR4 appear to be especially important in early activation of inflammation. Not only does TLR2/TLR4 signaling activate proinflammatory pathways in leukocytes, muscle fibers also express TLR2 and TLR4 which can activate expression of proinflammatory cytokines by muscle fibers, too (Fig. 10). Genetic ablation of TLR4 in *mdx* mice produced large reductions in numbers of intramuscular macrophages and fewer of the macrophages in the muscle expressed iNOS and more expressed CD206, reflecting a shift of macrophages away from the cytolytic, proinflammatory, M1-biased phenotype (109). Consistent with the reductions in muscle

membrane damage and improved muscle function in iNOS-null *mdx* mice (342), TLR4 ablation in *mdx* mice also reduced membrane damage and improved muscle function (109). Similarly, null mutation of TLR2 in *mdx* mice, reduced the numbers of macrophages in *mdx* muscle while decreasing the proportion of intramuscular macrophages that expressed iNOS and improving muscle function (218). Together, the findings suggest that the release of DAMPs from injured dystrophic muscle could activate the inflammatory response to dystrophic muscle and influence macrophage phenotype by acting through TLR2 and TLR4.

Although several DAMPs or other ligands may conceivably activate TLR2 and TLR4 signaling to initiate the inflammatory response to muscular dystrophy, experimental evidence shows that HMGB1 and fibrinogen are prominent candidates to serve this function. Fibrinogen is a large soluble serum protein that is expressed in the liver but is then proteolyzed at sites of injury to form the insoluble fragment, fibrin, that is necessary for clot formation (40). Thus, in injured tissue such as dystrophic muscle, there can be accumulations of fibrinogen and fibrin (both forms are collectively referred to herein as fibrin). Because fibrin is an endogenous ligand for both TLR2 and TLR4 (179, 225), its accumulation at high levels in the muscles of DMD patients and *mdx* mice can play important roles in influencing the inflammatory response to muscular dystrophy (341). Either genetic ablation of fibrin or pharmacological reduction of fibrin accumulation in *mdx* mice reduced muscle macrophage numbers while reducing pathology and improving function (341). Generally, this experimental outcome resembled the treatment effects of TLR2 or TLR4 ablation in *mdx* mice (109,218), suggesting fibrin and TLR2/4 may function through the same pathway to activate inflammation in *mdx* muscles. However, in contrast to the shift of muscle macrophages to a CD206+, M2-biased phenotype caused by TLR2 or TLR4 mutation (104), fibrin deletion in *mdx* mice reduced numbers of CD206+ macrophages (218, 341).

HMGB1 binding to TLR4 is also a potentially important mechanism for early activation of inflammation in muscular dystrophy. At early stages of *mdx* pathology, HMGB1 exits muscle fiber nuclei, where it normally resides in healthy cells, and accumulates in the cytoplasm, reaching supraphysiological levels (109). Presumably, the cytosolic HMGB1 is actively or passively released into the extracellular space following subsequent muscle fiber damage. The extracellular HMGB1 would then be available to bind its PRRs, including TLR2 and TLR4, to promote inflammation. This hypothetical mechanism is supported by the observation that treating *mdx* mice with glycyrrhizin, an inhibitor of HMGB1 binding, reduced inflammation and produced beneficial treatment effects that were similar to effects achieved by genetic ablation of TLR4 (109). Furthermore, treating TLR4 mutant *mdx* mice with glycyrrhizin produced no additive improvements in pathology, suggesting that HMGB1 and TLR4 act through the same pathway to activate inflammation in *mdx* dystrophy.

The findings summarized above support a general model in which mechanical damage to dystrophic muscle fibers causes a release of DAMPs from injured fibers that would then bind PRRs on inflammatory cells, expressed at highest levels by macrophages. Ligation of PRRs on macrophages, especially TLR4, would activate NFκB to drive the production of proinflammatory cytokines to amplify the inflammatory response and promote iNOS expression, leading to increased cytolysis. However, less direct, PRR-mediated events may

also lead to the production of proinflammatory molecules in dystrophic muscle that can activate and promote inflammation. For example, muscle cells themselves express intracellular PRRs that can bind endogenous DAMPs to increase production of proinflammatory cytokines. Muscle cells isolated from *mdx* mice express elevated levels of cell surface PRRs (TLR1, 2, 4) and intracellular PRRs (TLR3, 7, 8, 9), and showed increased release of the proinflammatory cytokine TNF and the potent inflammatory cell chemoattractant CCL2 following stimulation with either a TLR4 ligand (LPS) or TLR7/TLR8 ligand (ssRNA) (140). These findings show that muscle cells themselves may be direct respondents to extracellular or intracellular DAMPs that increase inflammation. Currently, the relative importance of PRR activation on muscle cells versus myeloid cells in driving early stages of inflammation in muscular dystrophy is unknown.

Because of the importance of the innate immune response in promoting the pathology of muscular dystrophy and the important role of TNF in regulating innate immunity, several investigations have explored whether ablation or blockade of TNF-mediated signaling would reduce the pathology of dystrophinopathy. Unfortunately, the findings show that systemic loss or reduction of TNF signaling can worsen some features of the *mdx* pathology, even while reducing muscle inflammation, and show that whether the interventions are beneficial or detrimental varies with the age of the animal and the tissue assayed. For example, genetic ablation of TNF worsened histopathology in the diaphragms of 4-week-old *mdx* mice, but had no effect on quadriceps pathology at 4 weeks of age, and then worsened quadriceps pathology in 8-week-old mice (296). Similarly, treating *mdx* mice with blocking antibodies to TNF caused reduction in inflammation at early stages of *mdx* pathology (21 days old) which was followed by higher levels of inflammation in treated *mdx* mice (25–26 days old), after which inflammation in treated *mdx* muscles fell below that occurring in untreated (28 days old) (129). This suggests that perturbing TNF signaling may affect the time course of the inflammatory response, and not just the magnitude of the response. Longer term ablation or blockade of TNF also shows mixed results. Although 10 to 12-month-old, TNF-null *mdx* mice showed improved respiratory function (122) and treatments with anti-TNF for 3 months improved treadmill running performance (263), treating *mdx* mice with anti-TNF for 6 months reduced cardiac function (86). Collectively, the observations reflect the complexity and unpredictability of the effects of systemic perturbations of an important signaling molecule, but also reflect the multitude of regulatory roles played by TNF, beyond regulating innate immunity.

**Amplification of the innate immune response—**Release of DAMPs from muscle appears to be important for initiating the innate immune response to dystrophic muscle and the ensuing recruitment of leukocytes is largely influenced by the subsequent release of proinflammatory cytokines and chemoattractant molecules from inflammatory cells and from muscle cells themselves. Although numerous chemoattractive molecules can contribute to the recruitment of immune cells to dystrophic muscle, signaling through CC chemokine receptor 2 (CCR2) has been definitively demonstrated as playing a significant role for recruiting inflammatory cells into *mdx* muscles. Disruption of CCR2-mediated signaling through either genetic or pharmacological interventions greatly reduced macrophage

numbers in *mdx* muscles, which was accompanied by reductions in fibrosis and improvements in muscle function (219).

Achieving the extremely high concentrations of innate immune cells in dystrophic muscle also requires induction of specific adhesion molecules that mediate myeloid cell migration into the damaged tissue, to follow chemoattractive signals. Although many cell surface adhesion molecules likely play significant roles in regulating the invasion of inflammatory cells into dystrophic muscle, osteopontin is now established as a potentially important molecule in this regard. Osteopontin is expressed by several cell types, including macrophages, T-cells, neutrophils, skeletal muscle cells, smooth muscle cells, endothelial and epithelial cells (37, 174, 231, 245, 328, 349). Following its release, it can function as a chemokine and mediate cell adhesion (247, 294, 300, 369, 380) by binding integrin molecules via arginine-glycine-aspartic acid (RGD) dependent and independent mechanisms (21, 96, 240, 337). These properties enable osteopontin to function as a proinflammatory molecule; for example, increases of osteopontin in acutely injured tissue increased the accumulation of macrophages at the injury site and the application of function-blocking antibodies to osteopontin reduced the effect (107).

As with other inflammatory mediators, osteopontin plays a beneficial role following acute muscle injuries but a detrimental role in chronic muscle damage, suggesting that its function and regulation are adaptive for the innate immune response to acute injuries or infection. Following severe, acute, muscle damage, osteopontin expression increases over 100-fold, peaking at 48 h postinjury, with macrophages being a primary site of osteopontin localization (144). Notably, the peak of osteopontin expression coincided with the early stage of muscle regeneration, suggesting that osteopontin may play a significant role in macrophage-mediated regeneration (144). Subsequent experimental evidence validated that possible regenerative role. Genetic ablation of osteopontin caused a significant delay in the accumulation of neutrophils and macrophages in acutely injured muscle and a slowing of muscle regeneration (327). However, the regulatory role of osteopontin in injured muscle is likely more than mediating myeloid cell adhesion to support their migration. Stimulation of macrophages with the full-length isoform of osteopontin, OPNa, caused elevated expression of chemokine ligand 5 (CCL5; a potent chemoattractant for leukocytes), IL-1β (a proinflammatory cytokine), IL-10 (an antiinflammatory cytokine), and tenascin C (201). Interestingly, tenascin C is a TLR4 ligand, suggesting an additional mechanism for TLR4 activation in injured muscle.

Despite the beneficial function of osteopontin in acutely injured muscle, its ablation produces a net reduction of pathology and functional improvements in *mdx* mice. Osteopontin expression in DMD (136) and *mdx* muscles (261) is tremendously elevated, with the protein located in macrophages in DMD muscle (375) and in macrophages and T-cells in *mdx* muscle (45, 340). The systemic ablation of osteopontin in *mdx* mice produced functional improvements and reductions in muscle fibrosis that were accompanied by reduced numbers of macrophages and neutrophils in *mdx* muscles but increases in CD4+ T-cells and FoxP3+ T-cells (45, 340). These findings support the interpretation that osteopontin's negative influence in chronically injured muscle is attributable to disruption of its immunomodulatory roles, including regulating myeloid and lymphoid cell migration.

However, osteopontin may also have a direct regulatory role in influencing myogenesis that may be impaired in chronic muscle injury. For example, osteopontin modulates the adhesion, migration, and fusion of myoblasts, at least *in vitro* (328) and chronic disruption of those functions by pathological levels of osteopontin could contribute to pathology. Stimulation of myoblasts *in vitro* with osteopontin also affects their expression of growth factors and cytokines that influence myogenesis (201, 328), which may have unexplored effects on the course of muscular dystrophy.

**Activation of the acquired immune response—**Although activation of an innate immune response is a direct and primary consequence of DAMP activated TLR signaling, there are also downstream effects on the acquired immune response that can affect immune cell interactions with dystrophic muscle. For example, activation of NFκB signaling following TLR ligation can promote the expression of TNF, IFNγ, and CCL2 (61, 287, 291, 329, 330), all of which can drive an innate immune response that is dominated by proinflammatory macrophages (Fig. 10); however, they can also promote a cellular immune response by increasing the activation and recruitment of CTLs (110, 120, 237, 293, 323, 377). Furthermore, the NFκB pathway also increases the expression of MHC class 1 and costimulatory molecules such as B7.1 (CD80), that are essential to activate the cellular immune response of CTLs to injured cells (97, 160, 167, 378). Thus, the inflammatory environment produced by TLR-driven activation of NFκB can support a cellular immune response to muscle fibers that express MHC class 1 on their surface, provided an antigen is presented on the fiber's surface in the context of MHC class 1 (Figs. 9 and 10).

Surprisingly, whether dystrophin-deficient muscle fibers express MHC class 1 on their surface is still disputed. Initial, immunohistochemical observations consistently noted expression of MHC class 1 on the surface of DMD fibers (9, 84, 209) and confirmed by protein assays that MHC class 1 levels were elevated in DMD muscles (209). In contrast, more recent investigators conclude that absence of MHC class 1 on the surface of DMD fibers can be used as a diagnostic tool to distinguish DMD from idiopathic inflammatory myopathies (IIM), in which MHC class 1 is expressed on the fiber surfaces (163, 335). However, other recent reports conclude that immunohistochemically discernible MHC class 1 is present on muscle fibers from DMD patients and IIM muscle fibers at similar levels (232) and show elevated expression of MHC class 1 on the surface of *mdx* muscle fibers (91). Although we do not know the basis for the differing conclusions regarding MHC class 1 on the surface of muscle fibers, a general consensus appears to be that not all fibers in DMD or *mdx* muscles express MHC class 1, with expression levels affected by stage of the disease (84, 209) and proximity of inflammatory cells to the muscle fibers (9, 232). Because of the small volumes of DMD muscle biopsy tissue used in the analyses, the relative prevalence of MHC class 1 expressing fibers may vary from sample to sample, depending on the site of biopsy and the stage of disease. In addition, previous history of treatment with corticosteroids can influence MHC class 1 expression (335) and previous corticosteroid use was typically an uncontrolled variable in the investigations and could have contributed to variable outcomes. Thus, the most parsimonious interpretation of the observations would be that DMD fibers can express MHC class 1 on their surfaces, although expression may vary

between individual patients and differ between muscle fibers and vary over the course of the disease.

Although the identity of the antigenic determinants that are presented with MHC class 1 on the surface of dystrophic muscle fibers is unknown, mutant dystrophin or proteins from the dystrophin-associated protein complex may be sources. Depending on the mutation of the dystrophin gene, the protein may be expressed but then more rapidly degraded than wild-type dystrophin. For example, in one particular missense mutation, leucine 54 in the actin-binding domain-1 of dystrophin was mutated to an arginine (L54R) which caused a reduction in dystrophin protein and produced a dystrophic phenotype (262). Despite causing a change in only a single amino acid, the mutation led to significant reductions in the tertiary stability of the mutant dystrophin, so that it was more rapidly degraded by the proteasome (308). The proteasome is a proteolytic complex comprised of α-subunits that are constitutively expressed and three pairs of catalytically active β-subunits that are variably expressed. In addition to expressing the standard proteasome present in all mammalian cells, antigen-presenting immune cells express an “immunoproteasome” that is distinguished by the inducible β-subunits LMP2, LMP7, and MECL. A major, specialized function of the immunoproteasome is the generation of MHC class 1-restricted, T-cell epitopes for presentation to CTLs (Fig. 9). However, skeletal muscles also express the immunoproteasome which can be upregulated following stress or injury (93,94,156) and is expressed at particularly high levels in *mdx* muscles (52). Thus, immunoproteasome-generated fragments of mutant dystrophin could provide a source for autoantigens to drive a cellular immune response to dystrophic muscle (Fig. 9). Similarly, DGC proteins are also targeted to the proteasome/immunoproteasome of dystrophic muscles. Because of the loss of dystrophin in DMD and *mdx* dystrophies, DGC proteins are less stable and more rapidly degraded through the proteasome, providing another source of potential autoantigens if their degradation occurs through the immunoproteasome.

Investigations of proteasome function in DMD and *mdx* muscles support a potentially significant role for immunoproteasome-generated antigens in contributing to the pathology of muscular dystrophy. For example, treating *mdx* mice with MG-132, a peptide aldehyde that inhibits the activities of the proteasome and calcium-dependent proteases and NFκB, increased the concentration of DGC proteins in *mdx* muscles, restored them to the cell membrane and reduced muscle membrane damage (34). Although these treatment effects could reflect the slowing of DGC proteolysis by the constitutive proteasome, MG-132 would also inhibit the immunoproteasome; thus, part of the rescue may reflect a reduction of a cellular immune response to dystrophic muscle. More recently, the immunoproteasome inhibitor ONX-0914, which is specific for the β-subunit LMP7, has been used to test that additional possibility (91). *Mdx* mice treated with ONX-0914 showed large reductions in muscle histopathology and significant improvements in muscle function. In addition, the large reductions in muscle membrane lysis were accompanied by significant reductions in numbers of CD4+ and CD8+ T-cells in the dystrophic muscles (91). Furthermore, the same treatment regimen applied to *mdx*/scid mice produced no improvements in muscle function or histology, indicating that the beneficial effects were mediated by actions on T-cells. However, the extent to which the reduction of T-cell-mediated pathology in the treated mice resulted from perturbing direct actions of the T-cells on the dystrophic fibers or resulted

from reductions in myeloid-cell-mediated pathology is unknown. The ONX-0914 treatment reduced expression of TNF and IFNγ, which could reduce activation of macrophages from a proinflammatory, cytolytic M1-biased phenotype to an M2-biased phenotype and feasibly contribute to the reduction in pathology. As shown in alveolar macrophages, LMP7 inhibition can increase macrophage polarization toward an M2-biased phenotype (54).

## Transitions in leukocyte populations during the progression of dystrophinopathies

**Macrophage populations undergo phenotype transitions as muscular dystrophy progresses—**An important distinction between the pathology of DMD and *mdx* dystrophies is that DMD is a continuously progressive disease while *mdx* muscles undergo a period of successful muscle regeneration between the ages of approximately 2 and 12 months of age, that is then followed by a progressive pathology. Several potential mechanisms may underlie the period of successful regeneration in *mdx* muscles. For example, the onset of *mdx* muscle regeneration coincides with an increase in expression of the dystrophin homolog, utrophin (199,206) which is normally expressed only in myotubes, neuromuscular junctions, and myotendinous junctions of healthy muscle (188, 243, 246). Upregulation of utrophin strengthens the *mdx* muscle cell membrane which is sufficient to greatly reduce the pathology of muscular dystrophy (70, 265, 321). Similarly, the onset of muscle regeneration in *mdx* muscle coincides with upregulation of another complex of structural proteins that includes vinculin and talin, which can functionally compensate for the loss of dystrophin and the DGC (188). That upregulation does not occur in DMD muscles (216).

Changes in macrophage phenotype may also contribute to the successful regeneration of *mdx* muscle that follows the acute onset of pathology. Although a population of iNOS-expressing, cytolytic, M1-biased macrophages dominates the early stages of *mdx* pathology, *mdx* muscle macrophages shift to a population of CD206-expressing, antiinflammatory, M2-biased macrophages at subsequent stages of the pathology (341, 342). M2-biased macrophages express elevated levels of arginase, IL-4, IL-10, TGFβ1, and insulin-like growth factor-1 (IGF-1), each of which can play important roles in reducing muscle damage. As noted above, competition between iNOS in M1-biased macrophages and arginase in M2-biased macrophages for their common substrate, arginine, reduces the production of cytolytic NO by iNOS, thereby reducing muscle fiber damage (342) (Fig. 6). Furthermore, IL-10 reduces the expression of iNOS in macrophages in *mdx* muscles which reduces their cytotoxicity and diminishes muscle membrane damage (343).

Macrophage transitions toward an M2-biased phenotype also enables them to promote muscle regeneration by acting on a population of muscle stem cells, called satellite cells. Satellite cells normally reside on the surface of muscle fibers in healthy muscle, enveloped by the basal lamina that surrounds each individual fiber (208). Following muscle injury, they are activated to proliferate, and some daughter cells proceed to differentiate to become new muscle fibers that can contribute to muscle regeneration (Fig. 11). Other daughter cells return to the quiescent satellite cell pool, awaiting the next round of activation (reviewed by ref. 81). However, over the course of muscular dystrophy, satellite cell numbers are depleted by the repeated rounds of muscle injury and repair, which may eventually impair the

regenerative capacity of dystrophic muscle. However, IL-10-stimulated macrophages increase proliferation of satellite cells, which could help maintain satellite cell populations needed for muscle regeneration (343) (Fig. 12). Elevations in IL-4 production also promote the transition of macrophages to an M2-biased phenotype (118, 119), in addition to increasing muscle repair by acting directly on satellite cells to promote their differentiation and growth (152). Similarly, IGF-1 secretion by macrophages in injured muscle can improve regeneration by acting directly on satellite cells to expand their numbers and simultaneously driving macrophages to the M2-biased phenotype, which has a higher capacity for promoting muscle growth (322). Subsequent growth of myotubes is also promoted by IGF-1, although fibroblasts appear to be the primary source of IGF-1 at that stage of regeneration (322).

Macrophages biased toward the M2 phenotype also promote growth and regeneration through a Klotho-mediated pathway. Klotho is a transmembrane protein from which the extracellular domain can be cleaved and released to function as a hormone or it can be expressed as a truncated form that is secreted (182, 193,207). Klotho has been studied most extensively in the context of aging because its mutation causes rapid changes in several organs that resemble premature senescence and its expression normally declines in multiple tissues during aging (182). Many of the physiological changes that accompany the loss of Klotho reflect its diverse roles in regulating vitamin D synthesis, regulating ion channels and transporters, and suppressing oxidative stress and growth factor signaling (153, 180, 181, 183, 332, 368).

Klotho is normally expressed in young, healthy muscle, but the loss of dystrophin from skeletal muscle causes epigenetic silencing of Klotho in muscle cells, which exacerbates muscle pathology (355, 358). However, as macrophages in dystrophic muscle transition toward a CD206+ M2-biased phenotype, they increase their expression of Klotho selectively at sites of muscle damage, which expands satellite cell populations and slows or prevents their loss over the course of the disease (Fig. 12) (358). In addition, elevations of Klotho in dystrophic muscle reduced muscle damage and fibrosis and increased longevity and improved motor function, indicating that it may function broadly as a macrophage-derived molecule that can reduce the pathology of muscular dystrophy (358). However, despite the physiological significance of macrophage phenotype transitions in the regeneration of dystrophic muscle, we do not yet know whether there are differences in the regulation of macrophage phenotype in DMD versus *mdx* muscles that may contribute to differences in the regenerative capacity of the diseased human and mouse muscles.

**Functions of CD4+ T-cells during the acute onset and regenerative stages of *mdx* dystrophy—**Early immunohistochemical investigations of DMD and *mdx* muscles showed that dystrophic muscles contained a relatively small population of T-cells that expressed the CD4 antigen (<1000 CD4+ cells/$mm^3$ muscle; 297). Most CD4+ T-cells are functionally classified as helper T-cells (Th cells) that are rich sources of cytokines that regulate the immune response by influencing the differentiation and activation of other immune cells. Th cells are also further designated as Th1 or Th2 cells, according to whether they primarily express proinflammatory cytokines (Th1 cytokines) or antiinflammatory cytokines (Th2 cytokines). Among the spectrum of cytokines produced by CD4+ Th cells

that can influence dystrophinopathies, IFNγ is the quintessential Th1 cytokine that can activate macrophages to a proinflammatory phenotype and increase their production of iNOS and can also activate CTLs. IL-4 and IL-10 are particularly important Th2 cytokines and, as noted above, they play central roles in regulating macrophage phenotype, as well as promoting macrophage-mediated muscle repair. IL-5, another Th2 cytokine, activates eosinophils (198,367) which can then increase damage of dystrophic muscle fibers and modulate the acquired immune response. Despite the complex and potentially antagonistic roles of CD4+ T-cells, experimental evidence shows that they serve a net detrimental role at early stages of *mdx* dystrophy. Antibody depletions of CD4+ cells from *mdx* mice that were analyzed at the acute onset of *mdx* pathology (between 3 and 4 weeks of age) showed large reductions in the muscle histopathology (297). However, whether the CD4+ T-cells then transition to a phenotype that promotes regeneration later in the course of the disease was not tested.

More recently, an additional population of CD4+ T-cells called regulatory T-cells (Tregs) has been identified in dystrophic muscle in which they reduce muscle inflammation and damage. Tregs differentiate from naïve T-cells that are activated by TGFβ which induces expression of the transcription factor FoxP3, the master regulator of Treg development. Generally, Tregs help maintain tolerance to self-antigens and are immunosuppressive, in part because of their expression of IL-10. The initial discovery of Tregs in *mdx* muscles implicated them in reducing pathology by showing a positive relationship between elevations in their numbers in rapamycin-treated *mdx* muscles and reductions in muscle fiber damage and histopathology (82). Furthermore, reductions in their numbers by antibody depletion of CD25+ cells from *mdx* mice increased muscle fiber damage, reflected by higher levels of serum CK (42), and increased muscle inflammation and IFNγ expression (344). The more specific ablation of FoxP3 expressing Tregs also worsened *mdx* muscle pathology and increased IFNγ expression, especially by CD4+ Th cells, and reduced the expression of the M2 activation marker, CD206, on macrophages (344) (Fig. 13). Conversely, increasing Treg numbers in *mdx* muscles reduced muscle pathology and reduced serum CK levels and increased IL-10 expression (344). Collectively, the observations indicate a significant regulatory role for Tregs in modulating immune cell functions in both cytolytic and regenerative functions in dystrophic muscle, despite their extraordinarily low frequency of occurrence, and show they play an important role in the transition from the early stage of *mdx* pathology to the regenerative stage. It is feasible that more successful regeneration of *mdx* muscles compared to DMD muscles could result, in part, from differences in Treg involvement in regulating immune cell involvement in muscle pathology. Although Tregs are present in DMD muscle (344), their potential functions have not yet been explored.

## The immune system promotes fibrosis of dystrophin-deficient muscle

**TGFβ1-mediated signaling in fibrosis of dystrophic muscle—**Although death of dystrophin-deficient muscle fibers that is caused by mechanical damage or by immune cells is the primary cause of muscle pathology in DMD or *mdx* dystrophies, muscle fibrosis that results from tissue damage and inflammation is an important, debilitating feature of the disease that contributes to increased mortality. As DMD and *mdx* dystrophies progress, fibrotic tissue continuously accumulates in muscle (257) which contributes to the loss of

ambulation, causes severely impaired respiration (301), and produces potentially lethal cardiac dysfunction (159, 217) (Fig. 14). Similar to other features of the pathology of DMD, the pathological fibrosis that occurs in dystrophinopathies is likely attributable to the misapplication of repair processes that are beneficial following acute injuries, but not during chronic damage. Following an acute injury, wound healing requires the production and remodeling of the extracellular matrix that supports and guides the growth and repair of damaged tissue. However, the same mechanisms applied to tissue that is chronically damaged over a lifetime can instead cause pathological fibrosis.

Much of the pathological fibrosis that occurs in DMD and *mdx* dystrophies is directly or indirectly attributable to the immune response to dystrophic muscle. As in many other diseases that involve tissue fibrosis, leukocyte-derived TGFβ1 has been implicated in the fibrosis of dystrophic muscles. TGFβ1 is pleiotropic protein that can regulate inflammation, fibrosis, cell proliferation, and cell differentiation. Although each of those potential downstream effects of TGFβ1 signaling could have important influences on the course and severity of muscular dystrophy, most investigations of TGFβ1 function in dystrophic muscle have focused on potential profibrotic effects that exacerbate pathology. That focus is largely attributable to preceding investigations which showed that elevated TGFβ1 expression increased production of connective tissue in several pathologies (68, 266, 272, 359) and the well-characterized pathway through which TGFβ1 induces the expression of major connective tissue proteins, such as fibronectin and collagens, through SMAD-mediated signaling (223). Early histological observations are consistent with a role for TGFβ1 in promoting fibrosis in DMD and *mdx* muscles. For example, TGFβ1 accumulates in the endomysium (28, 255, 370) and in mononucleated cells in the connective tissue (28) which agrees with the interpretation that leukocytes in DMD muscle express TGFβ1 that then accumulates in the connective tissue in a latent or active form. Subsequently, *in situ* hybridization data validated that TGFβ1 mRNA is present in endomysial inflammatory cells at early stages of *mdx* pathology, but also in regenerating fibers (379), perhaps reflecting a role for TGFβ1 in regeneration as well as fibrosis. More recently, bioinformatics analysis of networks of gene expression in DMD muscle showed that TGFβ-centered networks were strongly associated with muscle fibrosis (69), further supporting the involvement of TGFβ signaling in the pathological fibrosis of dystrophic muscles.

Elevated expression of TGFβ1 by leukocytes in *mdx* muscles is strongly influenced by the accumulation of fibrin in the damaged muscles, which has been linked to a pathogenic role for fibrin in the fibrosis of dystrophic muscle. Although the normal role of fibrin in blood clotting and wound healing is beneficial following an acute injury, the chronic and progressive accumulation of fibrin in injured dystrophic muscle worsens pathology. This has been shown by genetic ablation of fibrinogen and by treatment of *mdx* mice with a drug, ancrod, which disrupted fibrin deposition and produced reductions in fibrosis in *mdx* diaphragm muscles (341). Furthermore, ancrod treatments diminished the increase in TGFβ1 mRNA and protein and reduced collagen content in *mdx* diaphragms *in vivo* (341). Because inflammatory cells in lesions in *mdx* diaphragms are the sites of highest TGFβ1 expression (124) and fibrin stimulation of *mdx* macrophages *in vitro* increases expression of TGFβ1 mRNA and protein (341), the observations collectively support the conclusion that fibrin induces TGFβ1 expression by macrophages in inflammatory lesions, leading to

increased connective tissue accumulation in dystrophic muscle. The findings also revealed a potential feed-forward system through which fibrin binding by macrophages could further promote fibrosis. Both genetic and pharmacologic reductions in fibrin deposition in *mdx* mice significantly reduced numbers of M2-biased, CD206+ macrophages (341), possibly because TGFβ1 can influence the M2 phenotype. M2-biased CD206+ macrophages promote fibrosis in dystrophin-deficient muscles and hearts (352) and promotion of CD206+ macrophage populations through fibrin-activated signaling could increase fibrosis through TGFβ1-independent mechanisms (Fig. 12).

Although disruption of fibrin-activated TGFβ1-mediated signaling in *mdx* mice produced reductions in muscle fibrosis, whether diminishing TGFβ1-signaling in dystrophinopathies provides a strategy for long-term and beneficial treatments for muscular dystrophy remains uncertain. For example, DMD muscles show an elevation of TGFβ1 mRNA that peaks at 2 to 6 years of age and then declines, despite the progressive fibrosis that continues in DMD muscle for many years (28). That observation suggested that TGFβ1 plays a role in initiating but not perpetuating fibrosis in DMD muscle (28). Similarly, GRMD dogs that experience a progressive muscle pathology and fibrosis that resembles DMD, show an elevation of TGFβ1 mRNA expression in muscle only at early stages of the disease, that declines after 60 days of age while muscle fibrosis progresses (255). In addition, diaphragm muscles in *mdx* mice, which undergo a more rapid and continuously progressive pathology than *mdx* limb muscles, show elevated levels of TGFβ1 at 6 to 9 weeks of age, but no significant elevation at 3 months or 8 months of age (124, 379). Furthermore, *mdx* diaphragm muscles in 8-month-old mice do not express higher levels of TGFβ1 mRNA than age-matched limb muscles (238), although their rate of fibrosis is much greater. Nevertheless, even temporarily elevated expression of TGFβ1 in dystrophin-deficient muscle can lead to increased connective tissue deposition, at least in some muscles. Injections of *mdx* mice with neutralizing antibodies to TGFβ1 starting at 6 weeks of age produced significant reductions in endomysial connective tissue accumulation in diaphragms by 12 weeks of age (6) and administration of anti-TGFβ to *mdx* mice until 9 months of age produced reductions in connective tissue in the diaphragm and improved respiratory function (238). In contrast, treating *mdx* mice with neutralizing antibodies to TGFβ from 2 weeks to 9 months of age did not significantly affect fibrosis in the limb muscles that were assayed (238). Similarly, depletions of T-cells from *mdx* mice which can be rich sources of TGFβ produced large reductions in circulating levels of TGFβ, but had no effect on muscle fibrosis (224).

A potential explanation for the poor correlation between TGFβ1 expression and fibrosis of dystrophic muscle may lie in the requirement that TGFβ1 that is present in the connective tissue in a latent form must be proteolytically cleaved to become activated. Thus, increases in TGFβ1-driven fibrosis could occur in the absence of *de novo* synthesis of the protein, if there is progressive activation of previously expressed, latent TGFβ1 stored in the ECM. Although we do not know if this occurs in dystrophic muscle, several molecular mediators have been identified that could serve this role. For example, matrix metalloproteinase-9 (MMP9) cleaves and activates latent TGFβ1 (249, 374) and macrophages that invade *mdx* muscles are primary sources of MMP9 in the diseased tissue (192). In addition, genetic deletion of MMP9 is associated with a reduction of active TGFβ1 in *mdx* diaphragms (192).

As outlined in the preceding discussion, TGFβ1-mediated pathways can play significant roles in promoting fibrosis of dystrophic muscle, at least in some muscles and at least during early stages of the disease. In addition, leukocytes appear to be a primary source of TGFβ1 in dystrophic muscles, although regenerative fibers may also contribute. Surprisingly, we do not know which leukocyte populations are most important contributors to TGFβ1 production in dystrophic muscle. Macrophages, CD4+ T-cells and Tregs can also produce high levels of TGFβ1 and thereby modulate the immune response to dystrophic muscle in ways that will have influences beyond modulating fibrosis. For example, leukocyte-derived TGFβ1 can function as a chemoattractant to increase inflammatory cell populations (363), which influence the course and severity of pathology. TGFβ1 also plays complex roles in regulating macrophage function (32), that could affect growth and regeneration of dystrophic muscle. In addition, TGFβ1 is essential for induction of Tregs from Th populations by activation of SMAD signaling (89). If Tregs prove to play beneficial roles in DMD as observed in regenerating *mdx* muscles (344), then reductions in their induction by TGFβ1 could impair muscle regeneration.

Leukocyte-derived TGFβ1 can also play significant roles in regulating normal myogenesis, which is necessary for muscle regeneration. TGFβ influences the proliferation, differentiation, and chemoattraction of satellite cells (4, 5, 30, 302) and disruption of any of those satellite cell functions can profoundly affect muscle growth and regeneration. However, the net effect of inhibiting TGFβ signaling in dystrophic muscle may be beneficial to muscle function, regardless of whether there is a significant effect on fibrosis. For example, treatment of *mdx* mice with neutralizing antibodies to TGFβ for an 8.5 months period produced significant improvement in fore-limb muscle grip strength, restoring it to healthy wild-type muscle strength (238). Because the treatments did not reduce fibrosis in limb muscles, the functional improvements were likely not secondary to antifibrotic effects of anti-TGFβ1. Instead, the antibody treatments caused an increase in satellite cells expressing the transcription factor myogenin which is essential for muscle differentiation, suggesting that some of the treatment effects may reflect actions on myogenic cells (238).

**Myeloid cell interactions with fibrogenic cells in dystrophic muscle—**Because fibroblasts are major sources of connective tissue proteins, such as the collagens, and they secrete molecules that promote the production of connective tissue, such as connective tissue growth factor (CTGF), they have been presumed to be the primary cellular drivers of pathological fibrosis in muscular dystrophy. Early observations also indicated that close interactions between fibroblasts and inflammatory cells could influence the accumulation of connective tissue in dystrophic muscle. For example, the highest concentrations of fibroblasts expressing collagen type 1 or collagen type 3 in *mdx* muscles were within inflammatory lesions in muscles (114), sites that also contained the highest concentrations of immune cells, especially macrophages. More recent findings also show that at least some of the fibrotic cells that contribute to fibrosis in dystrophic muscle are derived from myeloid lineage cells that invade injured muscle and then differentiate into fibrocytes (347). Diaphragm and hind limb muscles from *mdx*$^{5cv}$ mice contained elevated populations of fibrocytes that expressed the leukocyte common antigen (CD45) and collagen type 1, in addition to F4/80, which is a marker of differentiated macrophages. Although the extent to

which these fibrocytes contribute to fibrosis in dystrophic muscle is unknown, the observation that fibrocytes in the more rapidly fibrotic diaphragm muscle expressed higher levels of extracellular matrix genes such as collagen types 1, 3, and 6 than expressed in fibrocytes from hind limb muscles, suggests that fibrocytes may play a significant role in the fibrotic process (347).

Although invading fibrocytes potentially contribute to pathological fibrosis in dystrophic muscle, a resident population of mesenchymal cells called fibro/adipogenic progenitor cells (FAPs) is now well-established as significant contributors to regulating the ECM in healthy, injured, and diseased muscle. FAPs, which normally reside in muscle in a quiescent state, are derived from a nonmyogenic lineage. However, they can influence myogenesis following injury or disease by modulating the microenvironment (166, 331). Muscle injury activates FAPs, causing their rapid proliferation followed by a rapid return to preinjury levels. During their normally brief period of activation, FAPs produce high levels of IL-6 and IGF-1, either of which can promote myogenesis (166). Following activation, FAPs may return to the quiescent state or die or differentiate into either adipocytes or fibroblasts (Fig. 7). Thus, perturbations that influence the postactivation state of FAPs can affect accumulation of fat or connective tissue in muscle and can influence the regenerative response of muscle to subsequent injuries, by determining whether sufficient numbers of FAPs return to the pool of quiescent cells.

Myeloid cells play a pivotal role in regulating the numbers and developmental fates of FAPs and, once again, regulatory processes that are adaptive to acute injuries are maladaptive to chronic muscle injuries, leading to worsened pathology. Following activation by acute muscle injury, expansion of FAP populations is negatively regulated by TNF that is released by M1-biased macrophages and causes FAP cell apoptosis (190). However, in the context of chronic muscle injuries with prolonged elevations of M2-biased macrophages, the normal regulatory effects of TNF on FAPs are disrupted. Individual macrophages in *mdx* muscle display an unexpected phenotype in which individual cells express TNF, which could reduce FAP numbers, but the same individual cells also express TGFβ which can block the induction of FAP apoptosis and increase collagen expression *in vivo* (190). Furthermore, *in vitro* observations show that wild-type macrophages induce FAP apoptosis (190), which shows that the reduction of FAP numbers by apoptosis is through a direct interaction between the two cell types. However, *mdx* macrophages failed to induce apoptosis of FAPs *in vitro*, indicating an intrinsic difference in *mdx* macrophages that affects their ability to regulate FAPs (190), which may contribute to the fibrosis of dystrophin-deficient muscles.

**Arginase metabolism in the fibrosis of dystrophic muscle—**Macrophages also increase fibrosis of dystrophin-deficient muscles by providing more substrate for the synthesis of connective tissue proteins, especially through the metabolism of arginine by arginase. In sterile inflammations in mice, arginase is synthesized by some M2-biased macrophages, where expression can be induced by IL4, TGFβ, and other Th2 cytokines (85, 104, 158). Hydrolysis of arginine by arginase produces ornithine which is then metabolized to generate proline that is necessary for the production of proteins in the collagen family which are rich in proline and hydroxyproline (17, 66). In injured tissue, the rate of connective tissue synthesis is influenced by availability of arginine and proline (17, 364,

365); thus, elevations in the numbers of arginase expressing cells in dystrophic muscle have the potential to accelerate fibrosis (Fig. 7). M2-biased macrophages that accumulate in *mdx* muscles express arginase-1 and arginase-2 at similar levels (352) and at least arginase-2 promotes fibrosis of *mdx* muscles. Genetic ablation of arginase-2 in *mdx* mice produced significant reductions in fibrosis of diaphragms and quadriceps muscles of 18-month-old mice, that were accompanied by reductions in scoliosis, a spinal deformity that is secondary to muscle fibrosis (352). However, the mutation did not reduce fibrosis in the soleus muscle or myocardium, which mirrors other muscle-specific and unexplained differences in the pathophysiology of muscular dystrophy. For example, extraocular muscles experience no pathology in *mdx* mice, triceps brachii experience more injury than other limb muscles and the diaphragm exhibits the most severe pathology (169, 301). Feasibly, the failure to reduce cardiac fibrosis by arginase-2 mutation in *mdx* mice may reflect a greater reliance on arginase-1 metabolism in cardiac macrophages. Although we have no direct evidence that M2-biased macrophages play similar profibrotic roles in DMD muscle as in *mdx*, CD206+ M2-biased macrophages in DMD muscle accumulate at sites of endomysial fibrosis in DMD muscle suggesting a possible functional relationship (75).

The profibrotic effect of elevated arginine metabolism by muscle macrophages can be further amplified by the secondary loss of neuronal nitric oxide synthase (nNOS) from dystrophic muscle. Muscle fibers from both DMD and *mdx* muscles express nNOS that is normally associated at the cytosolic face of the muscle cell membrane, in association with the DGC. However, with the loss of dystrophin and the reduction of the DGC in dystrophic muscle, nNOS expression is down-regulated which leads to a great reduction in NO generation by dystrophic muscle. Although loss of nNOS contributes to the pathology of muscular dystrophy because it perturbs normal homeostasis through multiple mechanisms (319), nNOS deficiency can also reduce competition between arginase and nNOS for their common substrate, arginine. The shift of arginine metabolism from NOS to arginase, called the “arginine switch,” can create a more profibrotic environment (282). A profibrotic environment can also be promoted by long-term, dietary supplementation with arginine. Although supplementing the diets of *mdx* mice with arginine or arginine injections for brief periods (2–4 weeks treatment) may reduce muscle histopathology and improve function (18, 145), dietary arginine supplementation of *mdx* mice for 17 months increases fibrosis of hearts, quadriceps, diaphragm, postural muscles and soleus muscles (352). Less extended periods of arginine supplementation show treatment outcomes can vary between muscles analyzed. Six months of dietary supplementation with arginine butyrate significantly increased diaphragm fibrosis, but decreased gastrocnemius collagen content without significantly affecting cardiac collagen content (130).

## The Immunobiology of Limb-Girdle Muscular Dystrophies

### Overview of limb-girdle muscular dystrophies

In the basic model that we present to relate the molecular defect causing DMD to the immune response that modulates the course and severity of the disease, we propose that the pathological involvement of the immune system is attributable, in part, to an innate immune response to acute cellular damage that is misapplied to a chronic injury. However, if we look

more broadly at the immune cell involvement in other muscular dystrophies, we see disparate immune responses to chronic damage. That diversity shows there is not a single immune response to chronic muscle damage and suggests that endogenous immunomodulators may contribute to diverse immune responses to genetically distinct muscular dystrophies. However, few of those endogenous immunomodulators have been identified. Some of the limitations of our knowledge are particularly evident in the family of diseases called limb-girdle muscular dystrophies (LGMDs); in some LGMDs, inflammation is a prominent feature of the pathology but little is known of the mechanisms regulating immune cell interactions with the dystrophic muscle, or even whether the immune system plays a beneficial or detrimental role.

LGMDs are a highly heterogeneous group of muscular dystrophies that can be caused by mutations in any one of over 20 distinct genes and can occur as either a dominant (LGMD type 1) or recessive (LGMD type 2) genetic disease and vary tremendously in severity, time of onset and clinical course. These muscular dystrophies are united into a single disease family based on the shared characteristic that they primarily affect muscles of the pelvic girdle and shoulder girdle, although muscles located more distally in the limbs can also be affected. Despite that clinical similarity, the mutations that cause the diseases occur in highly diverse genes, including genes that encode a structural/regulatory protein at the cell nuclear membrane (lamin A/C; LGMD1B), or a protease (calpain-3; LGMD2A), or proteins in the DGC (sarcoglycans; LGMD2C-2F) or a membrane repair protein (dysferlin; LGMD2B).

## Does the immune system play a role in LGMD1B or LGMD2A?

Clinical observations indicate that the immune system likely plays a significant but unexplored role in modulating the pathology of some LGMDs. In some of those patients, muscle inflammation is extensive and the patients are responsive to corticosteroid treatments, indicating that the immune cell involvement may have a net negative effect. For example, genetic analysis of 20 LGMD patients with a disease onset at 2 years or younger and who had been provisionally diagnosed as inflammatory myopathy showed that 11 of the patients had mutations in the lamin gene that encodes an intermediate filament protein at the nuclear membrane, and were therefore actually LGMD1B (175). All of the patients had elevated serum CK and half of the patients who received corticosteroids responded to the treatment. Furthermore, analysis of the intramuscular leukocyte population suggested an acquired immune response that included CD20+ B-cells and CD4+ and CD8+ T-cells (175) (Fig. 15). Nevertheless, we do not know how the immune system may be involved in LGMD1B. Similarly, prominent inflammatory infiltrates have been reported for subpopulations of LGMD2A patients, in which the disease is caused by mutations in the gene that encodes a calcium-dependent protease, calpain-3. In a cohort of 45 LGMD2A patients, the youngest patients with the shortest disease duration showed prominent muscle inflammation in contrast to older patients who showed no inflammation but extensive fibrosis (276) (Fig. 16). In addition, the inflammatory infiltrate in the younger group was enriched in eosinophils. Despite the provocative relationships between disease stage and inflammatory involvement and the unusual inflammatory infiltrate, we do not know whether the immune system is involved in the pathophysiology of LGMD2A.

## Does the immune system have a significant influence on the pathology of sarcoglycanopathies?

Several diseases that are grouped into the LGMD family are caused by mutations to genes that encode one of four transmembrane components of the DGC, called sarcoglycans. Mutation of any one of the sarcoglycan genes can cause disruption of the entire sarcoglycan complex and affect the DGC, producing defects in cell structure and signaling pathways. Disease onset typically occurs in childhood and involves muscle weakness and muscle mass loss. Unlike DMD, sarcoglycanopathies usually do not cause prominent respiratory muscle dysfunction or cardiac pathology. However, like DMD, sarcoglycanopathy patients experience elevated serum CK, showing the occurrence of muscle membrane damage or breakdown.

Although histological assessment of muscle biopsies from sarcoglycanopathy patients frequently show muscle inflammation, little is known about mechanisms through which the immune system may influence the onset, course or severity of any of the sarcoglycanopathies. For example, in the mouse model of LGMD2E, in which there is a mutation in the gene encoding β-sarcoglycan (βSCG), there is prominent elevation of macrophage numbers in limb and diaphragm muscles, fibrosis and a transient elevation of TGFβ (108), but the role of the inflammatory cells is unknown (Fig. 17). Skeletal muscles in the mouse model of LGMD2D, in which α-sarcoglycan is mutated, also show elevated numbers of CD11b+ leukocytes and elevated levels of serum CK (39). Treatments of LGMD2D mice with HCT1026, a potent antiinflammatory drug and nitric oxide releasing molecule, reduced inflammation, improved function, and reduced muscle membrane damage (39), suggesting a potential cytolytic role of leukocytes in LGMD2D. Similarly, treatment of γ-sarcoglycan mutant mice (LGMD2C) with the immunomodulatory drug FTY720 reduced muscle inflammation and reduced muscle membrane damage (141) supporting the possibility that leukocytes may play a net, negative role in sarcoglycanopathies. However, specific cellular and molecular mediators through which the immune system may affect the sarcoglycanopathies are not known.

## Overview of dysferlinopathies

In contrast to other LGMDs, muscular dystrophies that are caused by mutations of the dysferlin gene are among the best characterized in the context of muscle interactions with the immune system over the course of the disease and illustrate that there is not a single immune response to chronic muscle membrane damage. Dysferlinopathies are autosomal recessive diseases that are characterized by slowly progressive, muscle wasting and weakness that primarily affects limb muscles (Fig. 18). Dysferlinopathies are caused by mutations in the dysferlin (DYSF) gene and patients with dysferlinopathies may be identified as afflicted with limb girdle muscular dystrophy 2B (LGMD2B) or Miyoshi myopathy, according to the clinical presentation (196). The dysferlin gene encodes a protein that mediates repair of damaged cell membranes (16, 19, 20, 23, 256). Normally, cell membrane microlesions can be repaired rapidly by mechanisms that involve transport of dysferlin to the injury site where its calcium-dependent interactions with membrane phospholipids contribute to resealing the lesions (16, 49). In the absence of dysferlin or the presence of mutant dysferlin, membrane repair can be defective, leading to leaky cell

membranes. Although dysferlin is expressed in numerous tissues, the pathophysiological consequences of its deficiency are most apparent in muscle, presumably because frequent and extreme mechanical stresses on muscle cell membranes lead to more membrane lesions that require repair. In addition, the primary defect that initiates the pathology of dysferlin-deficiency lies in muscle, not other cells. This was clearly demonstrated by experimentation in which the muscle specific expression of a dysferlin transgene in dysferlin-deficient mice (Dysf(−/−) A/J mice) provided complete rescue from all pathological features of dysferlinopathy, including preventing macrophage invasion of the muscle (215).

The persistent membrane lesions in dysferlin-deficient muscles are likely the primary defect in dysferlinopathies. Predictably, concentrations of the muscle cytosolic protein, muscle CK, are elevated in the serum in dysferlinopathy, reflecting the persistent damage to the muscle cell membranes (135). Although in some extreme cases serum CK values in dysferlinopathy can reach values that surpass 20, 000 units/liter and can exceed serum CK levels in DMD (135, 170), disease severity is less and disease onset is later in dysferlinopathies than in DMD. Life expectancy in dysferlinopathy is in the normal range and disease onset is typically between 20 and 30 years. The greater longevity is attributed to less cardiac and respiratory muscle involvement in dysferlinopathies than in DMD. However, the less severe limb muscle pathology in dysferlinopathies compared to DMD, despite the more extensive membrane damage, shows that factors other than the extent of damage to the sarcolemma are important in regulating disease severity in muscular dystrophies.

Just as in DMD, the onset of pathology in dysferlin-deficient muscles in humans and in mouse genetic models is associated with an immune response to tissue damage. Also, as in dystrophin-deficient muscle, increased susceptibility of dysferlin-deficient muscle fibers to mechanical damage can provide the trigger for subsequent inflammation (273, 274) which may possibly lead to increases in damage caused by oxidative stress (314). Immunohistochemical analysis of leukocyte populations in muscle biopsies of dysferlinopathy patients showed the inflammatory infiltrates contained CD4+ and CD8+ T-cells and macrophages (57, 62, 372) and that the total numbers of inflammatory cells in dysferlinopathy muscles and DMD muscles were similar (62). However, whether the inflammatory infiltrate in dysferlinopathy muscles is dominated by macrophages is disputed, with macrophage: T-cell ratios reported as either 0.7:1 (102) or 3.6:1 (62) or as 0.5:1 (57) (Fig. 19). In each case, CD8+ CTLs were scarce in dysferlinopathy muscle biopsies and were typically found in the perimysium and not near muscle fiber surfaces (57, 62, 102) suggesting they may not play an important cytolytic role, although this has not been tested experimentally. Mouse models of dysferlinopathy experience a similar inflammatory infiltrate, that is more definitively dominated by macrophages. For example, the SJL/J mouse in which a splice-site mutation in the dysferlin gene lead to reductions in dysferlin (31) shows a slowly progressive muscle pathology with an inflammatory infiltrate that consists of CD4+ T-cells, CD8+ T-cells, and macrophages, but macrophages are the predominant cell type at each stage of the disease (239). In addition, muscle fibers in SJL/J mice display MHC class 1 on their surfaces, although genetic ablation of the β2-microglobulin gene in SJL/J mice produced a deficiency in muscle MHC class 1 levels without reducing histopathology (177). Generally, these features of the cellular infiltrate of dysferlin-deficient muscle support the expectation that immune cell involvement would

resemble that which occurs in DMD; however, accumulating evidence shows striking differences between immune cell involvement in the two diseases.

## Does dysferlin-deficiency in macrophages influence the pathology of dysferlinopathy?

A complicating aspect for our developing understanding of the pathophysiology of dysferlinopathies is that macrophages also express dysferlin and any systemic mutation of dysferlin can affect macrophage response and interactions with diseased and injured muscles. Dysferlin is synthesized in human, peripheral blood leukocytes that express CD14, a TLR4 coreceptor on the surface of monocytes, macrophages and neutrophils (71, 146). Upon differentiation, monocyte expression of dysferlin is further elevated (74) and macrophages that reside in the endomysium of healthy muscle also express high levels of dysferlin (72). Furthermore, dysferlin is distributed at the monocyte cell surface in association with integrin β3 (74), a transmembrane protein that mediates cell adhesion, suggesting a possible modulatory role for dysferlin in monocyte/macrophage interactions with the ECM.

Dysferlin-deficiency in monocytes/macrophages perturbs several functions that may potentially affect the normal inflammatory response to muscle damage. For example, monocytes isolated from the peripheral blood of LGMD2B patients showed less adhesion to substrates *in vitro* and displayed greater mobility and velocity of cell movements (74). Those observations supported the expectation that dysferlin-deficiency in monocytes/macrophages *in vivo* would yield an inflammatory population that would invade injured tissue more rapidly and perhaps at greater numbers. However, the similar numbers of macrophages in DMD and LGMD2B muscles suggest that loss of dysferlin from monocytes/macrophages does not tremendously amplify their invasion into injured muscle in humans. Furthermore, transplantation of wild-type mouse bone marrow cells expressing a green fluorescent protein (GFP) reporter or transplantation of dysferlin-mutant bone marrow cells that expressed GFP into dysferlin mutant mice resulted in nearly identical numbers of GFP+ cells in the recipients' muscle at 2, 7, and 10 months posttransplantation (14). This provides strong, *in vivo* evidence that invasiveness of monocytes/macrophages into dysferlin-deficient muscle is not influenced by dysferlin expression in leukocytes, at least in mice.

Dysferlin-deficiency in leukocytes may also affect their ability to phagocytose cellular debris in diseased muscle, which could slow or prevent muscle repair and regeneration. However, once again, *in vitro* and *in vivo* observations are not in complete agreement on this point. For example, *in vitro* assays of phagocytosis by monocytes isolated from SJL/J mice showed that loss of dysferlin increased phagocytic activity of monocytes (233), suggesting that the monocyte specific deletion of dysferlin would affect the cells' ability to phagocytose and remove debris in injured tissue. In contrast to that expectation, SJL/J mice experiencing severe acute injury that was caused by injection of snake venom experienced less inflammatory cell invasion and slowed clearing of necrotic debris compared to injured wild-type muscles (55). Although that finding could reflect a reduction in phagocytosis in acutely injured dysferlinopathic muscle, the reduction could also have resulted from fewer phagocytes in the muscle rather than deficiencies in phagocytosis at the cellular level. In addition, whether the inflammatory response to muscle injury caused by snake venom

mimics the response to muscle dysferlin deficiency has not been tested. Finally, analysis of *in vivo* phagocytosis of nanoparticles that were injected into wild-type or dysferlin-deficient muscles showed no differences in the proportion of macrophages that ingested particles between the two strains, although the total number of macrophages in mutant muscles was greater than in wild-type (333). Those observations show that there is not a significant difference in phagocytosis by dysferlin-deficient and wild-type macrophages, at least for nanoparticles in the context of wild-type versus dysferlin mutant mouse muscles.

## Regulating leukocyte invasion into dysferlin-deficient muscles

Although the signals that drive inflammatory cells to invade dysferlin-deficient muscles are unknown, several investigations suggest a mechanism in which activation of TLRs on leukocytes may contribute to their activation and recruitment to the diseased muscles. For example, genetic deletion of MyD88, a TLR-associated adapter protein, in dysferlin-mutant mice reduced the invasion of inflammatory cells into dysferlin-mutant muscles that had been injected with ssRNA, a DAMP and TLR ligand (326). Although unknown whether dysferlinopathic muscle releases ssRNA to attract leukocytes, the findings support the potential signaling mechanism. Likely, the ssRNA activation of TLR/MyD88 signaling occurred through TLR7 or TLR8, both of which are ssRNA receptors and were significantly upregulated in ssRNA injected muscles (326). However, other MyD88 signaling mechanisms must also contribute to leukocyte recruitment to the diseased muscle because MyD88 ablation caused only an insignificant trend for fewer inflammatory cells in dysferlinopathic muscle that had not been injected with a DAMP (326).

Other non-DAMP proteins may also initiate TLR signaling in dysferlinopathies, with thrombospondin-1 (TSP1) being a particularly strong candidate for this function. TSP1 is expressed by several cell types, including skeletal muscle, monocytes, macrophages and fibroblasts (73, 77, 161, 162, 227), after which it is deposited in the ECM, binding to any of several matrix proteins, including fibronectin (309). TSP1 functions in wound-healing are well-characterized, where it can promote fibrosis by cleaving and activating TGFβ1 (65, 285), and regulate leukocyte activation and recruitment (334). In addition, TSP1 is a TLR4 ligand that binds TLR4 and activates TLR4 and NFκB signaling, at least in BMDMs (194). Interestingly, differentiated myotubes that are dysferlin null mutants, or in which dysferlin expression has been reduced by siRNA, express more TSP1 than wild-type myotubes (73). Furthermore, treating dysferlin mutant myotube cultures with neutralizing antibodies to TSP1 greatly reduced their chemoattraction of monocytes. Notably, the elevated expression of TSP1 is directly related to dysferlin's absence and is not simply attributable to degeneration because undamaged, wild-type myotubes expressed more TSP1 when dysferlin expression was silenced by siRNA (73). These observations suggest the interesting possibility that the immune cell infiltrate and immune cell interactions with injured muscle in dysferlinopathies may be unique, because of a disease specific elevation of TSP1 that then functions as a significant endogenous immunomodulator.

**NFκB signaling in dysferlin-deficient muscle and macrophages—**The central role of NFκB signaling in regulating inflammation and myogenesis suggests that it may play a significant role in the pathophysiology of dysferlinopathies. Muscle biopsies from dysferlin-

deficient human muscles showed elevations in NFκB protein and signaling, as well as an increased nuclear localization of activated NFκB p65, indicating an increased induction of NFκB target genes (269). In addition, cytokines that activate NFκB signaling (e.g., TNF, IL1β) and transcripts and proteins that are promoted by NFκB signaling (e.g., TNF, IL1β) are elevated in dysferlinopathic muscles (59, 60, 76). Elevations of these cytokines would support a proinflammatory environment in which macrophages would be biased to the proinflammatory M1 phenotype, in agreement with the reported prevalence of M1-biased macrophages in dysferlin-deficient mouse muscles (14). Elevated expression of NFκB targets in wild-type macrophages can be driven by soluble factors released from dysferlin-deficient muscle cells *in vitro*. Conditioned media from dysferlin-deficient (A/J mice) muscle cell cultures caused increased expression of TNF and iNOS, in contrast to conditioned media from wild-type muscle cells that increased TGFβ production by macrophages (14). Although it was not tested explicitly whether the increased production of TNF and iNOS was attributable to elevated NFκB signaling, the findings are consistent with the interpretation that soluble factors released specifically by dysferlin-deficient muscles increase NFκB induction of genes encoding proinflammatory and cytolytic molecules. Furthermore, the finding that dysferlin-deficient muscle cells experience more necrosis and apoptosis than experienced by wild-type muscle cells when they are cocultured with M1-biased macrophages (14), suggests that the mutant muscles would be especially susceptible to cytotoxic mechanisms driven by M1-biased macrophages.

Despite the strong *in vitro* evidence indicating that the activation of proinflammatory pathways in macrophages can increase death of dysferlin-deficient muscle, whether the net effect of inhibiting inflammation in dysferlinopathy will reduce muscle damage is disputed. For example, the corticosteroids deflazacort and prednisone reduce DNA binding by NFκB and thereby reduce the expression of proinflammatory TNF while increasing expression of antiinflammatory IL-10 by leukocytes (186), but a clinical trial of deflazacort treatments of dysferlinopathy patients for 1 year showed a trend for increased muscle weakening compared to placebo control, which recovered after discontinuation of the drug (346). However, the effects of deflazacort treatment on inflammatory involvement were not assayed and deflazacort may have affected many physiological processes other than inflammation that could have contributed to a negative outcome. Similarly, dysferlin-deficient A/J mice that received celastrol, an inhibitor of NFκB activation, for 4 months showed no functional improvement although inflammation was reduced (76). Collectively, the findings indicate that broadly suppressing inflammation in dysferlinopathy may not provide a useful therapeutic strategy, although proinflammatory macrophages have the capacity to kill dysferlin-deficient muscles. More specifically targeted immune interventions will be needed.

## Activation of the complement system contributes to the pathology of dysferlin-deficient muscle

The pathology of dysferlinopathies is strongly affected by activation of a complex component of the innate immune system, called the complement system. More than 30 proteins that include serum proteins and cell surface receptors comprise the complement system, which is adapted to kill foreign or damaged cells, to activate phagocytes that remove cellular debris and to modulate the inflammatory response. Most of the soluble, serum

components of the complement system are present as inactive monomers. However, injury or infection can activate proteases that cleave inactive complement proteins to form biologically active fragments. Those fragments can then self-assemble through any of three potential pathways to form a membrane-attack complex (MAC) that can cause lysis and death when assembled in the cell membrane of a target cell (226, 284). Other proteolytic fragments that are generated by complement activation can modulate the inflammatory response, which can reduce infections but can also increase tissue damage in sterile injuries. For example, C3a and C5a are activated complement fragments that can increase binding by circulating myeloid cells to the vascular endothelium, an interaction that is necessary for immune cells to exit the vasculature into injured or diseased tissue. C3a and C5a are also potent chemoattractants for leukocytes and they can increase the production of proinflammatory cytokines and free radicals by myeloid cells (48,83,115,116). Thus, the complement system is adaptive for rapid responses that kill and remove infectious or diseased cells, but chronic activation of the complement system has the potential to increase tissue damage.

Prolonged activation of the complement system is a prominent, consistent feature of dysferlinopathies. In an analysis of biopsies from 14 human dysferlinopathy patients who experienced an age of disease onset that ranged from 3 to 43 years, all patients showed specific localization of the MAC (C5b-9) at the surfaces of muscle fibers (372) (Fig. 19F). In contrast, muscle fibers from DMD patients showed no detectible MAC or little MAC at the fiber surfaces (372). Significantly, the entire surface of nonnecrotic muscle fibers from dysferlinopathy patients showed MAC deposition (286, 299, 360), indicating that MAC may play a role at early stages of muscle pathology, before the initial events in fiber death are apparent. Experimental data also validate a significant role of the complement system in dysferlinopathies. Dysferlin mutant mice that were also null mutants for C3, a central component of the complement system, showed less histopathology than dysferlin mutant mice that expressed C3 (133). Interestingly, *mdx* mice that were C3 mutants did not show reductions of pathology, compared to *mdx* mice, indicating some specificity for the complement system in the pathology of dysferlin-deficiency (133).

Despite the prevalence of the MAC at the surface of dysferlin-deficient muscle fibers and the contribution of the complement system to the histopathology of dysferlinopathies, we do not know how the complement system is activated in dysferlin-deficient muscle. The three pathways for complement activation, the classical, alternative and lectin pathways, overlap at some regulatory steps such as the cleavage and activation of C3 to form C3a and C3b and each pathway culminates in MAC formation. The classical pathway is involved in humoral immunity and is activated by C1 binding to antigen-antibody complexes. However, there is no evidence of IgM or IgG deposition on the surface of dysferlin-deficient muscles, so this pathway is likely not involved. The lectin pathway is activated by mannose-binding lectin to mannose or other sugars frequently found on the surfaces of bacteria and viruses. Currently, there is no evidence that the lectin pathway is activated in muscle injury or disease. The alternative pathway can be activated by the spontaneous hydrolysis of C3 which is normally present at high concentrations in sera, exceeding 1.0 mg/mL in sera, and can be activated in muscle as a result of muscle damage through mechanical loading or ischemia (80, 99, 278),

suggesting that the alternative pathway is a likely, but unproven, route for complement activation in dysferlinopathies.

Part of the explanation for why the complement system may be a significant component in the pathology of dysferlinopathies, but apparently not a major factor in the pathology of dystrophin-deficient muscle may lie in differences in how complement activation is regulated in the two diseases. CD55, also called decay-accelerating factor or DAF, is a membrane bound protein that is a negative regulator of the complement system and indirectly inhibits the formation of the MAC (222, 251). This constitutive, low level of negative regulation is functionally important because there is a constant low level of C3 activation via the alternative activation pathway even in healthy tissues, which makes the system primed for a rapid response to infection or injury; loss of that negative regulation could result in an uncontrolled amplification of complement activation, leading to tissue damage (360). However, in dysferlin-deficient humans and mice, CD55 expression levels and protein levels in muscle are significantly reduced, which could provide an intramuscular environment that favors complement-mediated damage to the host tissue (233, 360). The likelihood that this CD55 deficiency could be functionally significant was supported by the finding that *in vitro*, primary myotubes from LGMD2B patients were more sensitive to complement mediated lysis than were healthy control myotubes and antibodies to CD55 increased myotube death (360). This potentially important mechanistic insight concerning the role of CD55 deficiency in dysferlinopathies may also help our understanding of why cardiac muscle is little affected by dysferlin-deficiency. RNA analyses showed that although CD55 expression was reduced in SJL/J skeletal muscles compared to controls, its expression was elevated in SJL/J hearts, and immunohistochemical observations showed no difference in apparent quantity of CD55 in SJL/J hearts versus healthy controls (360). Together, the findings indicate that CD55-deficiency may be a significant endogenous immunomodulator, contributing to disease specific features of dysferlinopathies.

## The Immunobiology of Congenital Muscular Dystrophies

### Overview of congenital muscular dystrophies

As shown through previous discussion of DMD and LGMD2B, mutations in genes that encode intracellular proteins that maintain the strength and integrity of the muscle cell membrane can cause defects that lead to the increased release of intracellular proteins into the extracellular space. Those released molecules can include DAMPs that activate an immune response that amplifies pathology. Similarly, mutations in genes that encode transmembrane or extracellular matrix proteins that function in muscle cell interactions with the ECM can produce structural defects in the sarcolemma, release of cytosolic proteins into the extracellular space and muscular dystrophy, but whether those mutations lead to a functionally important immune response to the pathology is more equivocal. Many of those genetic diseases are grouped into the congenital muscular dystrophy (CMD) family. In this section, we present evidence that suggests that the immune system may play a role in the pathophysiology of some CMDs.

The CMDs consist of at least 30 different genetic diseases with a combined frequency of occurrence that is estimated to be about 1:22,000. Although the clinical features of CMD

pathology are highly variable, patients characteristically show muscle weakness at birth or with a clinical onset during the first neonatal months, frequently accompanied by joint contractures. In some CMDs, muscle pathology primarily affects limb-girdle muscles, which can lead to the disease designation as LGMD. Serum CK levels are frequently highly elevated in CMDs and muscles show necrosis and fibrosis. Remarkably, despite the large number of genetic diseases that comprise the CMDs and the variability of clinical and pathological features, the great majority of gene products affected by CMD mutations are proteins that influence interactions between muscle and the extracellular matrix. Those proteins can serve as direct mechanical links that provide structural functions or they mediate posttranslational modification of those structural proteins, especially through their glycosylation.

Although there are functional relationships between many of the deficient gene products in CMDs, whether their deficiency leads to an immune response to the diseased muscle varies greatly. For many CMDs, there is no reported immune involvement in the pathology. For others, inflammation appears to be an important contributor to the disease. In a broad sense, many features of the inflammation of CMD muscles resembles inflammation in DMD and LGMD2B; the inflammatory infiltrate is typically dominated by myeloid cells, especially macrophages, it accompanies muscle membrane damage and corticosteroids or other immunosuppressive drugs can be beneficial, in at least some cases. These similarities suggest there may be common features in the involvement of the immune system in some of the rare CMDs as occur in more common muscular dystrophies that have better characterized inflammatory involvement.

## Lamininopathies

Among the CMDs, muscular dystrophies that result from mutations in the laminin-2 gene lead to the most extensive and best characterized inflammatory response to the disease. Laminin-2, also called merosin, is an important structural protein in the connective tissue sheath (basement membrane) that surrounds each muscle fiber and forms an interface across which all mechanical and chemical signals must pass to or from the muscle. Laminin-2 in skeletal muscle consists of three polypeptide chains, α2, β1, and γ1, and binds to the muscle fiber via interactions between the laminin-α2 chain and α-dystroglycan, a member of the DGC. Approximately 30% of all CMD cases result from mutations in the LAMA2 gene that encodes the laminin-α2 chain, causing in humans the CMD designated as MDC1A (Fig. 20). The well-characterized muscular dystrophy that occurs in the *dy* mouse strain also results from mutation of the mouse *Lama2* (366) causing a pathology that resembles MDC1A in humans.

The severe pathology that occurs in MDC1A is typically accompanied by elevated serum CK, as high as 150-times normal (165), and occasionally by severe inflammation (137, 258). However, the inflammatory cell infiltrate in muscle in most MDC1A biopsies is unremarkable to moderate, which has suggested that the immune response is typically not a major component of the pathophysiology. An alternative or additional explanation for the inconsistent involvement of inflammatory cells may be that the immune response is specific to stage of pathology, so that biopsies which show no inflammation may have been taken at

an immune silent stage of disease. For example, serial biopsies of a patient who was confirmed as having a loss-of-function mutation of the laminin-α2 gene showed a very prominent inflammatory infiltrate at 5 months of age, but little inflammation at 9 months of age (258). This trend for higher levels of inflammatory involvement in younger MDC1A patients that diminished with age was reported to be a general characteristic of laminin-α2 deficiency, and was accompanied by an age-related reduction in serum CK levels (258). Further exploration of the identity of cells in the inflammatory infiltrate showed elevated numbers of B-cells, and CD4+ and CD8+ T-cells (258), suggesting there may have been an acquired immune response to the diseased muscle at early stages of pathology. However, despite the inflammatory involvement in some MDC1A biopsies, whether the immune system has a significant effect on the course or severity of MDC1A pathology is unknown.

Although the potential involvement of the immune system in the pathology of human MDC1A has not been explored in depth, observations on laminin-α2 deficient mice provide evidence that the inflammatory response to the mutant muscle may increase pathology, especially by promoting fibrosis. As observed in MDC1A humans, laminin-α2 deficient mice show an early and rapid invasion of muscle by immune cells that consist primarily of monocytes and macrophages (149, 164, 211, 348) and eosinophils (164). Furthermore, the early onset of inflammation is accompanied by elevated levels of TLR2, TLR4, TNF, CCL2, and IL-1β and increased NFκB activation (164, 211, 348) indicating that the early immune response is dominated by a proinflammatory Th1 response. This cellular infiltrate has the unexplored potential to increase muscle fiber damage, but may also influence muscle fibrosis, which is a feature of laminin-α2 deficiency. Treating laminin-α2 mutant mice with molecules that block the angiotensin II type 1 receptor (AT1) reduced both muscle fibrosis and macrophage numbers in laminin-α2 mutant mice (1, 212). Because AT1-mediated signaling produces TSP1 which activates latent TGFβ1, the results indicate that TGFβ1 plays a significant role in fibrosis of laminin-α2 mutant muscle. However, whether leukocytes are the source of the TGFβ1 is unknown.

The innate immune system can also promote muscle pathology in laminin-α2 mutant mice through activation of the complement system. Ablation of C3 in laminin-α2 deficient mice increased longevity in the mutant mice, reduced muscle macrophage numbers and provided a transient protection against loss of muscle strength as the diseased progressed (63). These investigators also showed that oral administration of corticosteroids increased longevity of laminin-α2 mutant mice, which further implicates the inflammatory response in the pathology of laminin-α2 deficiency. Although corticosteroid treatments of MDC1A patients has also been reported to increase muscle strength (88), this has not been tested in depth.

## Dystroglycanopathies

Dystroglycanopathies are a collection of muscular dystrophies that result from mutations of genes that encode the dystroglycans or encode enzymes that affect dystroglycan glycosylation. The dystroglycans are central components of the DGC that are essential for normal interactions between muscle fibers and the surrounding extracellular matrix. Most basically, they provide a link between the actin cytoskeleton, via dystrophin, and extracellular structural proteins, especially laminin-α2 (Fig. 2). Because dystroglycans

(DGs) are part of the DGC, they are greatly diminished at the muscle cell membrane in DMD or *mdx* dystrophies. Thus, part of the pathology of DMD may reflect a reduction of extracellular α-DG and its transmembrane binding partner, β-DG. The ability of the DGs to interact normally with ECM proteins such as laminin-α2, is greatly influenced by glycosylation status of α-DG. Normal binding of α-DG to extracellular ligands involves at least six known or putative glycosyltransferases: protein O-mannosyl transferase 1 (POMT1), protein O-mannosyl transferase 2 (POMT2), protein O-mannose b-1, 2-N-acetylglucosaminyltransferase (POMGnT1); fukutin, fukutin-related protein (FKRP) and LARGE. The heterogeneity of dystroglycanopathies reflects the large number of potential genetic targets that can contribute to perturbations in α-DG interactions with the ECM.

Null mutations of the dystroglycan gene are not known to cause pathology in humans, probably because the mutations are early embryonic lethal; mice that are dystroglycan null mutants die very early in embryonic development (362). However, point mutations of the dystroglycan gene that affect glycosylation sites of α-DG can cause dystroglycanopathies (134). In addition, mutations in the genes encoding any of the validated or putative glycosytransferases that glycosylate α-DG can cause hypoglycosylation of α-DG and deficient binding to laminin-α2, resulting in muscular dystrophies that can vary greatly in severity and time of onset. In some cases, mutations of one of the six glycosyltransferases can cause severe pathology at birth or early infancy, leading to their categorization as Walker-Warburg syndrome (WWS), which is a CMD. In addition to muscle weakness and elevated serum CK, WWS and other early-onset dystroglycanopathies involve developmental delay with mental retardation because of lost function of α-DG in brain development. Mutations in POMT1, POMT2, fukutin, FKRP, and LARGE with α-DG hypoglycosylation have been associated with WWS (24,25,67,197,338,339). However, in other cases, mutations of one of the six glycosyltransferases produce a relatively mild, later onset pathology with primary involvement of limb girdle musculature (LGMD2I) (38).

Some of the most severe cases of dystroglycanopathies involve tremendous inflammation that can lead to an initial misdiagnosis as an inflammatory myopathy. For example, biopsy of one 6-year-old myopathic patient with severe muscle weakness, highly elevated serum CK and psychomotor developmental delay showed massive inflammation consisting primarily of macrophages, CD4+ T-cells and CD8+ T-cells (185). Although the magnitude of inflammation resembled an inflammatory myopathy, other pathological features indicated a dystroglycanopathy, which was confirmed by demonstrating the absence of a glycosylated epitope on α-DG (185). This patient also had point mutations in the FKRP gene, upstream of exon 1, which may have been involved in the pathology, although no mutations in the coding region of the gene were detected.

Typically, dystroglycanopathies are associated with little or moderate inflammation but the possibility that immune cells influence the pathology is largely unexplored. However, some clues suggest that immune involvement in these diseases may be significant in some cases. For example, two siblings with clinical onset of hypotonia at 4 months of age and a phenotype resembling LGMD were found to have immune cell infiltrates in muscle biopsies that were primarily macrophages and CD4+ and CD8+ T-cells (113). Genetic screening showed that both patients harbored fukutin mutations. Surprisingly, both patients also

showed remarkable improvements in muscle function following treatment with corticosteroids, that was reversed when steroid treatments stopped (113), suggesting that immune cells in the affected muscles exacerbated the muscle disease and weakness. Similarly, a patient diagnosed with CMD, with elevated serum CK and muscle inflammation consisting primarily of macrophages, was identified as harboring a mutation in POMT2 (29). When treated with corticosteroids, the patient's serum CK levels declined, but returned to high levels following discontinuation of corticosteroid treatment (29), implying that a significant portion of muscle damage was attributable to inflammation. Inflammation is also a prominent feature in a mouse model of dystroglycanopathy (myd mice) in which there is a spontaneous mutation of the LARGE gene, causing α-DG hypoglycosylation (125, 204). However, whether immune cells affect pathology in myd dystrophy is unknown.

## The Immunobiology of Facioscapularhumoral Muscular Dystrophy

### Overview of facioscapularhumoral muscular dystrophy

Throughout much of the preceding discussion, an underlying theme is that in muscular dystrophies in which there is damage to sarcolemma, an ensuing inflammatory response can exacerbate the pathology. Predictably, many of the deficient gene products that cause muscular dystrophies that show a significant inflammatory component are involved in maintaining the integrity of the sarcolemma or are necessary for normal interactions between the sarcolemma and surrounding ECM. A provocative and perplexing exception to that simple model is provided by facioscapularhumoral muscular dystrophy (FSHD), in which there is a failure to epigenetically silence a transcriptional activator, called DUX4. According to a current view of DUX4 involvement in FSHD, the protein is normally expressed in germline cells, but fails to be silenced in the skeletal muscles of FSHD patients, leading to activation of numerous genes that are normally involved in stem-cell-like transcriptional programs (311). Genes that encode proteins involved in apoptotic programs are among those genes activated by DUX4 and skeletal muscle apoptosis is one of the prominent features of FSHD, which may be partially responsible for the pathological progression of the disease (178, 345).

The consequence of DUX4 expression in skeletal muscle cells is a progressive muscle weakness that initially affects muscles of the face, scapula, and humerus, with later involvement of pelvic, thigh, and abdominal muscles (Fig. 21). FSHD onset is typically before 20 years of age and is slowly progressive; although life-span is nearly normal, increasing muscle weakness can lead to wheelchair dependence. Serum CK levels are typically moderately elevated (155). Approximately 30% to 40% of FSHD patients experience muscle inflammation (35), with immune cells appearing at especially elevated levels in the endomysium and near blood vessels (11,46). The inflammatory infiltrate is enriched with macrophages, CD8+ T-cells and CD4+ T-cells (11, 101, 150, 229, 248) (Fig. 21), suggesting a cellular immune response, perhaps to muscle fibers and the vasculature.

### Do immune cells influence the pathology of FSHD?

The question of why the immune system responds to muscle expressing DUX4 is perplexing and unanswered. However, a recently offered speculation links the expression of genes

normally expressed in germline cells in skeletal muscle to a potential autoimmune response (311). Because germline cells are immunologically privileged, proteins that they express are not seen by the immune system and do not invoke an immune response. However, the ectopic expression of those proteins, for example, in skeletal muscle, would make them available for immune surveillance, in which case they could be seen by the immune system as foreign antigens and activate a cellular immune response to the cell. Although there is no direct experimental evidence to address this potential, autoimmune mechanism as a component of FSHD, MHC class 1 occurs on the surface of some FSHD fibers (11) which supports the possibility that those fibers present autoantigenic peptides to CTLs or other immune cells.

Not only are the processes that drive the immune response to FSHD muscle unknown, whether inflammation has a net-positive or net-negative effect on pathology has not been established. Early observations showed a correlation between the extent of muscle necrosis and the number of inflammatory cells in FSHD muscles (11) and showed that the magnitude of muscle damage indicated by serum CK concentrations was greater in patients in which the FSHD involved inflammation (220). However, those relationships could reflect a role for immune cells in either amplifying pathology or reflect an increase in inflammation as a consequence of greater levels of tissue damage. Frequently, a positive response to corticosteroids reflects a net negative effect of inflammation on muscular dystrophies, but the findings on FSHD are mixed. In one case study of four patients from four separate families, each of whom experienced muscle inflammation, all the patients responded well to corticosteroids (229) although the beneficial effects were transient (228). In contrast, another case study of eight FSHD patients who showed evidence of inflammation in muscle biopsies showed no improvement in muscle strength after 12 weeks of corticosteroid therapy (310).

Despite the absence of functional data to show an influence of the immune response to FSHD on the magnitude or course of pathology, several features of the inflammatory involvement in the disease resemble aspects of the immune response to DMD or LGMD2B that promote pathology. FSHD patients show increased, systemic elevation of numerous proinflammatory cytokines that can increase pathology in other muscular dystrophies. For example, systemic levels of TNF and IFNα2 were elevated in all FSHD patients examined and CCL2, IL1α, and IFNγ were elevated in many patients (67%, 40%, 33%, respectively) (325). In addition, RNA analyses show that expression of many genes in the complement system are upregulated in FSHD muscles (267) and the MAC has been identified on the surfaces of nonnecrotic muscle fibers in FSHD (299), suggesting complement activation may be an early feature in cytolysis of FSHD by the innate immune system. Although these observations indicate that DUX4 expression in skeletal muscle would have a proinflammatory effect and would increase activation of the innate immune response, other *in vitro* findings indicate that DUX4 overexpression in muscle may also have antiinflammatory effects. Overexpression of full-length DUX4 in skeletal myoblasts increased expression of β-defensin 3, which can inhibit the expression of proinflammatory genes in macrophages (106). Clearly, the immunobiology of FSHD is a new frontier and its exploration may reveal surprising new insights into interactions between the immune system and diseased muscles.

## Conclusions

For many decades, muscular dystrophies were distinguished from inherited inflammatory myopathies by the supposition that the immune system did not play a significant role in the pathology of muscular dystrophy. Discoveries beginning in the 1990s have now shown that for many muscular dystrophies, especially DMD, the secondary immune response is an important determinant of the magnitude and course of the disease. That realization is exciting not only because it provides new insights into pathogenic mechanisms in the muscular dystrophies and provides new understandings into how muscle and the immune system interact, it also introduces new therapeutic targets for the treatment of muscular dystrophies.

As we learn more about the immune response to chronic damage of dystrophic muscle, we see that there are important differences between the responses to genetically distinct muscular dystrophies, even if they share key pathogenic features such as chronic lesions to the muscle cell membrane. Those differences show that there are disease-specific consequences of each genetic defect that affect the interactions between muscle and the immune system. In a very few instances, disease specific immunomodulatory effects have been identified. For example, the finding that silencing dysferlin in muscle cells increases expression of an important immunomodulatory molecule, TSP1, through an unknown mechanism (73) may convey some disease specific features to immune cell function in dysferlinopathies. Similarly, the discovery that dysferlin mutations cause a reduced expression of CD55 (DAF) (360), an important negative regulator of complement activation, may impart an increase in complement-mediated killing of muscle fibers that is specific to dysferlinopathies. Likewise, the disease specific silencing of nNOS expression in DMD muscle (50) may contribute to the relatively high and persistent levels of inflammation seen in dystrophin-deficient muscles compared to many other muscular dystrophies. Undoubtably, as we learn more about disease specific perturbations in gene expression in the muscular dystrophies, changes in the expression of additional immunomodulatory genes that contribute to the particular pathological features will be identified.

Perhaps specializations in the immune response to dystrophic muscle extend beyond differences attributable to distinct mutations or even beyond idiopathic differences between individuals with the same disease. For example, why are there marked differences in the magnitude of inflammation between different muscles in muscular dystrophy? Are those differences the consequence of differences in muscle damage, differences in the expression of immunomodulatory molecules generated by different muscle groups or differences in the susceptibility of different muscles to invasion or damage by leukocytes? Even within individual muscles, these same questions arise. Inflammation occurs in foci within muscles, that are sharply delineated from healthy, noninflamed sites. Again, are differences between individual muscle fibers or groups of fibers attributable to differences in injury, signaling or susceptibility to leukocyte invasion?

Advancing our understanding of the regulatory interactions between the immune system and dystrophic muscle has already led to identifying new potential therapeutic strategies, beyond the use of nonspecific immunosuppressants such as prednisone, the pharmacological

workhorse for many muscular dystrophies. For example, more specific interventions that rely on targeting activation of the complement system or the activation of NFκB or involve affecting the function of Tregs or myeloid cells are currently under exploration and development. Although developing new immunotherapeutic strategies will not cure any of the muscular dystrophies, they may provide effective new approaches to managing the pathology. However, even beyond potential therapeutic advantages of improving our understanding of the immune response to muscular dystrophy, these continuing studies are providing us with deeper and more thorough knowledge of the basic immunobiology of muscle and the importance of those interactions in maintaining health and homeostasis.

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## Didactic Synopsis

**Major teaching points**

1. Inherited muscular dystrophies arise from diverse mutations that lead to pathologies that have the shared characteristic of muscle wasting.
2. Many of muscular dystrophies, especially the most commonly occurring muscular dystrophy called Duchenne muscular dystrophy (DMD), cause a more easily damaged cell membrane that contributes to muscle death.
3. Damage muscle fibers in DMD and other muscular dystrophies can produce an immune response that can amplify muscle pathology.
4. The initial immune response to DMD muscle entails an ancient response called innate immunity, which is adaptive for acute injuries but is maladaptive for chronic muscle damage that occurs over the lifetime of the affected individual.
5. Perturbations in the expression or activity of endogenous immunomodulators can significantly influence interactions between muscle and the immune system that are specific to different muscular dystrophies.
6. The immune response to dystrophic muscle extends beyond innate immunity in which myeloid cells are the primary effector population, to include components of the acquired immune system, in which the actions of lymphoid cells are of central importance.

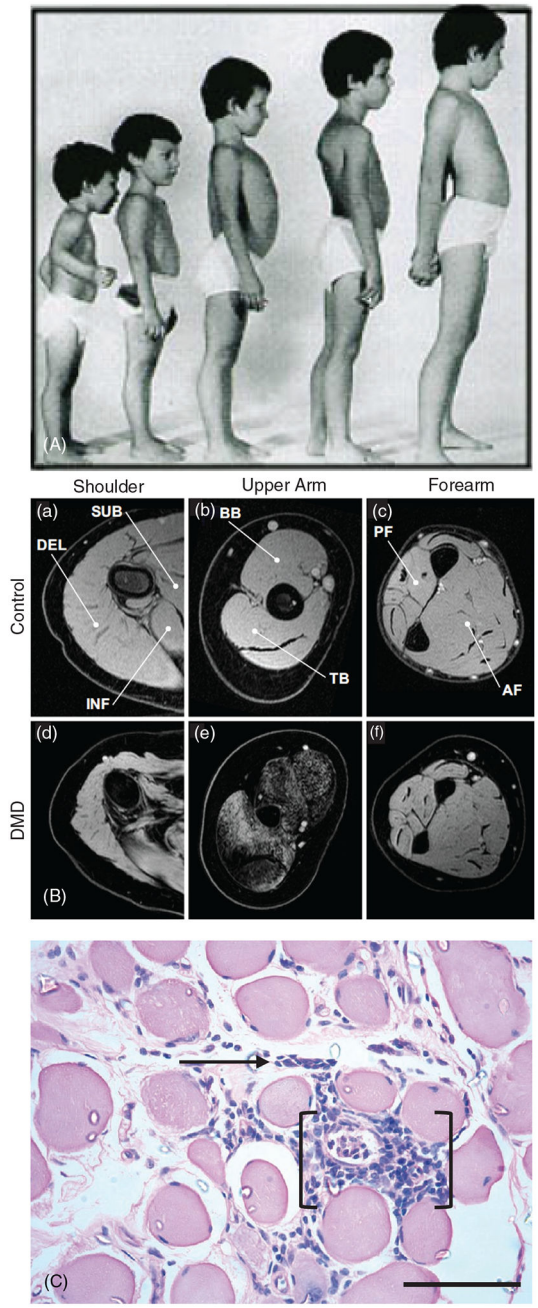



**Figure 1.**

Characteristics of Duchenne muscular dystrophy (DMD). (A) Typically, the clinical onset of DMD occurs at about 3 years of age as boys begin to show defects in muscle function. In this image of 5 boys with DMD at progressively older ages, some of the gross features of the disease are apparent. The boys show an increasingly progressive lumbar curvature of the spine that results in postural compensation for increased weakening of paravertebral muscles. There is also a progressive increase of weight bearing on the toes and reduction of weight bearing on the heels, as fibrosis of calf muscles cause contractures that limit plantar flexion. Although gross appearance shows an apparent sparing of calf muscles even in older

boys, there is actually an increased replacement of muscle tissue with fibrous and fatty tissue, leading to a pseudohypertrophy of the calves. (B) Magnetic resonance spectroscopy images of the shoulders, upper arms, and forearms of healthy subjects (12, 13, and 13 years of age) and DMD patients (12, 13, and 13 years of age) showing tremendous reductions of muscle mass (white) by fatty tissue (dark). BB (biceps brachii), TB (triceps brachii), DEL (deltoid), subscapularis (SUB), infraspinatus (INF), posterior compartment of the forearm (PF), and anterior compartment of the forearm (AF). [Reproduced, with permission, from reference (361).] (C) Section of muscle biopsy of DMD patient stained with hematoxylin and eosin stain. Large accumulations of connective tissue separate individual muscle fibers and mononucleated leukocytes are present in elevated numbers in the interstitial tissue (arrow) and near blood vessels (brackets). Fiber cross-sectional area is also highly variable, another characteristic of DMD pathology. Bar = 60 μm. [Reproduced, with permission, from reference (252).]

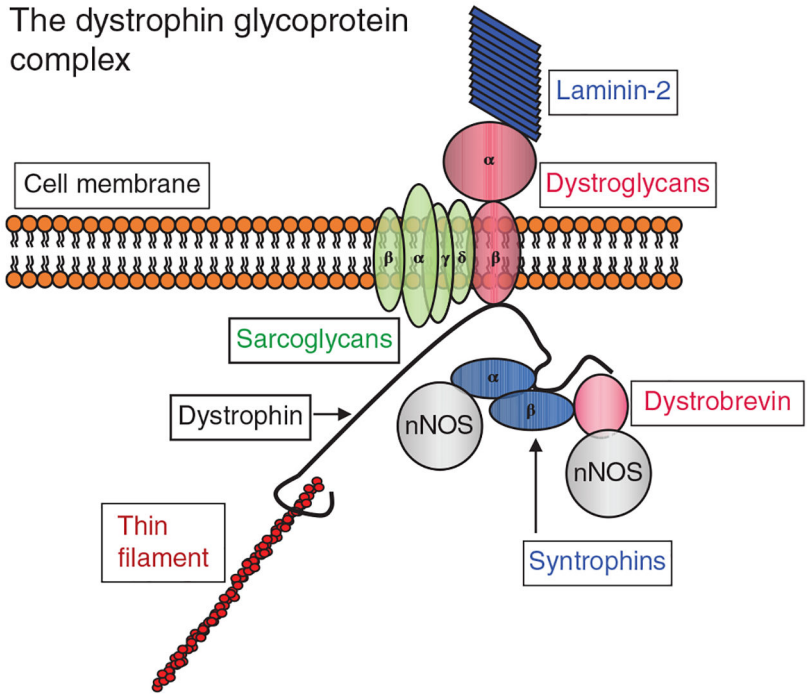



**Figure 2.**

Schematic representation of dystrophin glycoprotein complex (DGC). Dystrophin provides an important structural link between the thin filaments within skeletal muscle fibers and β-dystroglycan, which is a transmembrane protein. β-dystroglycan then binds extracellularly to α-dystroglycan which is a ligand for extracellular structural proteins, especially laminin-2 that is present in basal lamina. Several proteins in the basal lamina, including laminin and fibronectin, then provide mechanical links to major connective tissue macromolecules such as collagen type 1. Genetic deletion of dystrophin disrupts this mechanical linkage between the cytoskeleton and extracellular structural proteins, but also leads to reductions in the quantity of other structural proteins in the DGC or associated with the DGC. The secondary loss of those other proteins, including the sarcoglycans, syntrophin and neuronal nitric oxide synthase (nNOS) can contribute to the pathology of dystrophin deficiency through disruption of signaling pathways that are necessary for normal muscle function. [Reproduced, with permission, from reference (319).]

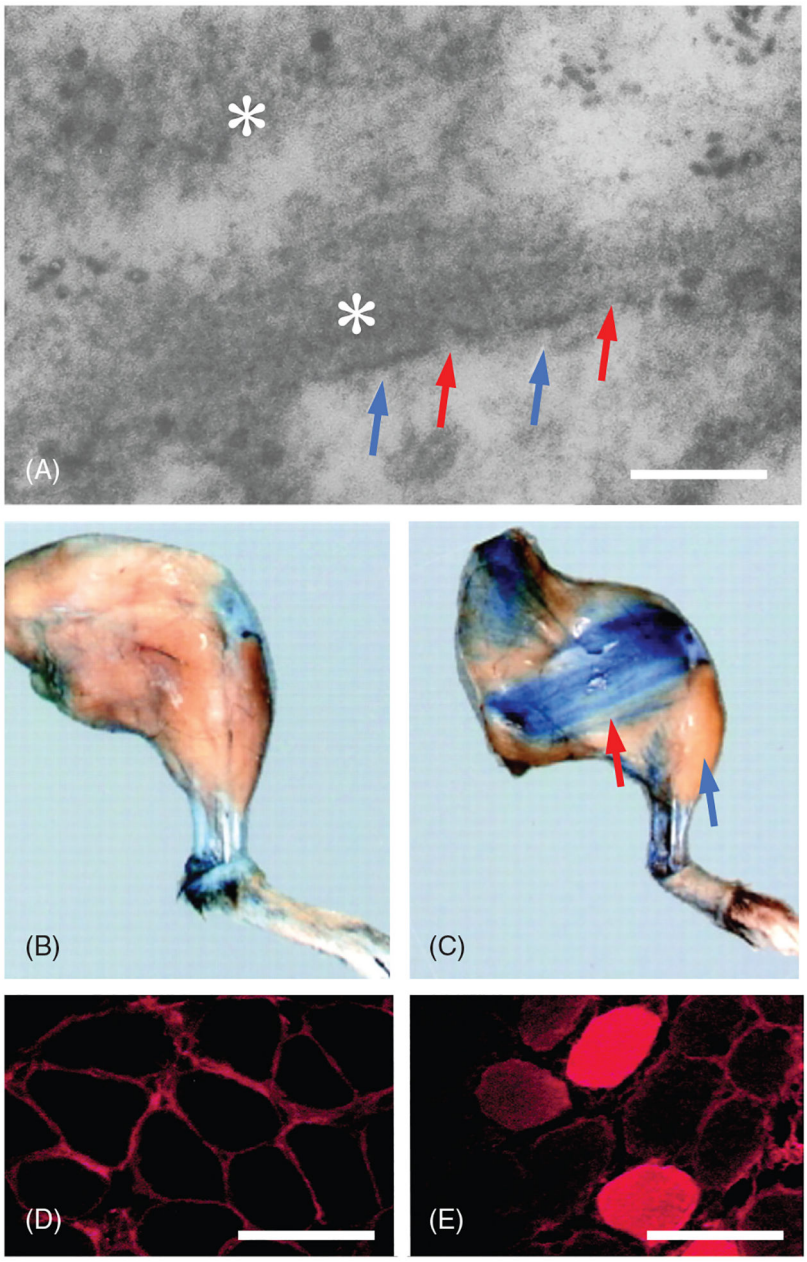



**Figure 3.**

Dystrophin deficiency causes membrane lesions and unregulated influx and efflux of large molecules. (A) Electron microscopy on DMD skeletal muscle shows morphologically detectible lesions in the cell membrane (sarcolemma) of dystrophic fibers. Asterisks (*) indicate basal lamina and other connective tissue associated with the extracellular surface of the muscle fiber. Blue arrows indicate sites of intact sarcolemma associated with dense, subsarcolemmal material. Red arrows indicate sites of lesions in the sarcolemma that would permit unrestricted transit of molecules across the membrane. Scale bar = 150 nm. [Reproduced, with permission, from reference (230).] [(B) and (C)] Intact hind limbs of wild-type (B) or *mdx* mice (C) that had been injected with an extracellular marker dye,

Evans blue, prior to euthanasia and tissue collection. Skin and fat has been removed from both limbs. The wild-type muscles show little blue dye, indicating that little dye was able to cross the cell membrane to enter the cytosol of the muscle fibers. The *mdx* muscle shows some thigh muscles contain high concentrations of blue muscle fibers (red arrow) indicating unregulated entry of dye into the fibers through membrane lesions. In contrast, leg muscle in the same mouse shows little blue dye (blue arrow), illustrating the difference in magnitude of pathology and progression of the disease in different muscles. [Reproduced, with permission, from reference (303).] [(D) and (E)] Cross sections of soleus muscle from wild-type (D) and *mdx* (E) mice. The muscles were incubated in a fluorescent, extracellular marker dye after dissection and before freezing the muscle for histology. In the wild-type muscle, the fluorescent dye remains in the extracellular space because the muscle cell membranes are intact (D). In the *mdx* muscle, approximately 8% of the muscle fibers showed elevated intracellular fluorescence, indicating the presence of membrane lesions. Bars = 70 μm. [Reproduced, with permission, from reference (351).]

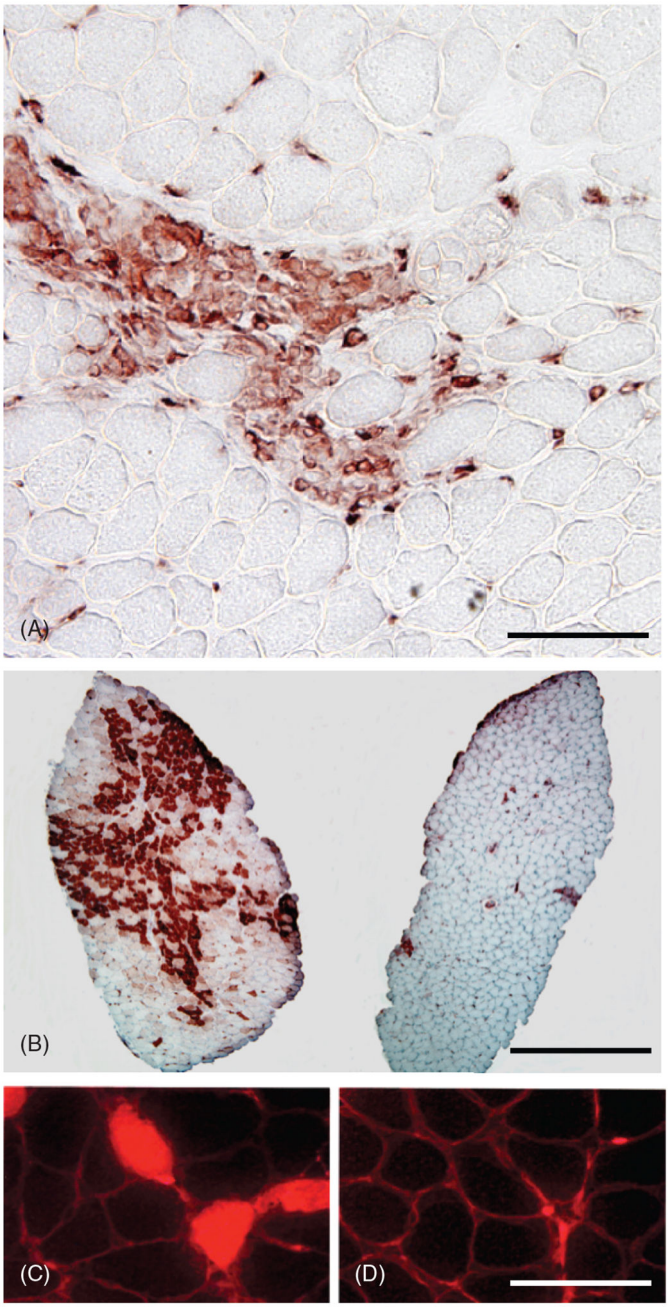



**Figure 4.**

Macrophages are a primary source of muscle fiber damage in the *mdx* model of DMD. (A) At the early, acute peak of *mdx* muscle pathology, macrophages dominate large inflammatory lesions in the diseased muscle. Cross section of 4-week-old *mdx* mouse muscle with F4/80 + macrophages immunolabeled to appear red in the sectioned muscle. Bar = 180 μm. (B) Image shows complete, mid-belly cross section of an entire soleus muscle from 4-week-old *mdx* mice. The mouse from which the muscle on the left was collected received intraperitoneal injections of sterile buffer on 5 days each week from 8 days of age until the mouse was euthanized at 4 weeks of age. The mouse from which the muscle on the

right was collected received intraperitoneal injections of antibodies to the F4/80 antigen on the same injection schedule, which produced a reduction in macrophage numbers by over 90%. Both sections were stained with antibodies to neural cell adhesion molecule (NCAM) which is expressed at high levels by muscle fibers that are undergoing repair following injury. Each small, red tile-like structure in the cross section is a recently injured fiber. Note that the macrophage-depleted muscle is nearly devoid of NCAM-expressing muscle fibers. Bar = 1.2 mm. [(C) and (D)] Soleus muscles from nondepleted, 4-week-old *mdx* mice (C) or macrophage-depleted *mdx* mice (D) were incubated in Procion red, a fluorescent, extracellular marker dye before muscle sectioning and microscopy. Intracellular Procion red indicates fibers with membrane lesions. The number of Procion-red-containing fibers in the 4-week-old *mdx* solei was reduced by more than 75%. Bar = 120 μm. [Reproduced, with permission, from reference (351).]

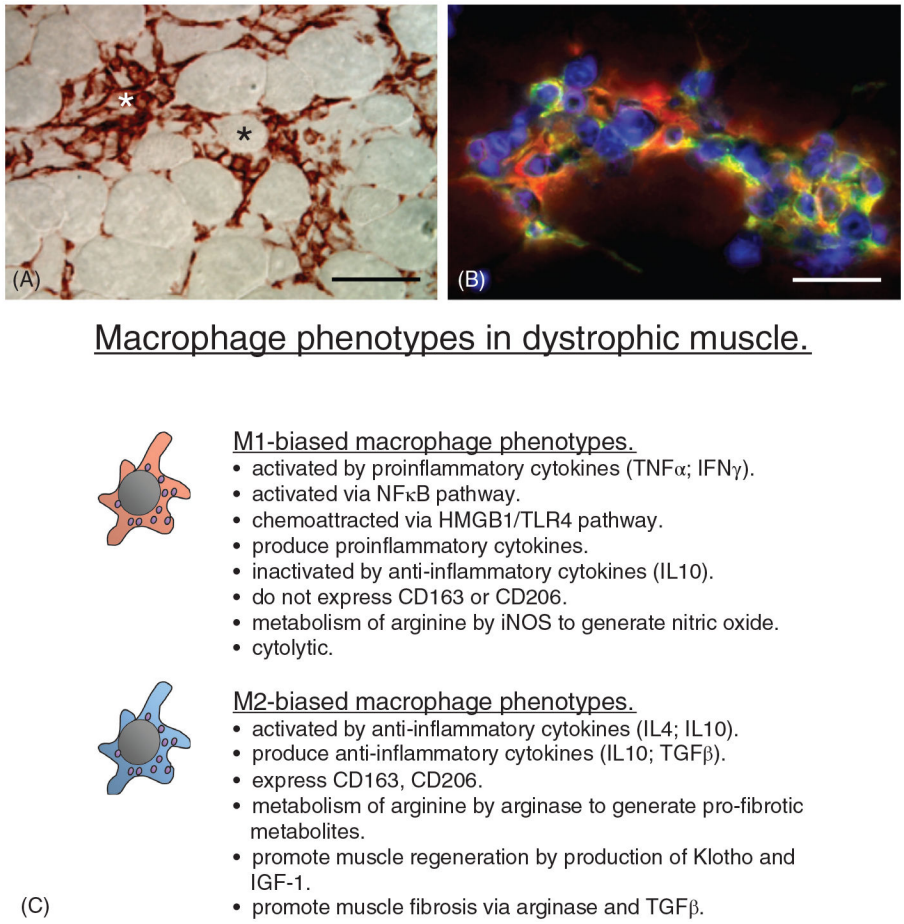



**Figure 5.**

Inflammation in the *mdx* mouse model of DMD. (A) Section of a 4-week-old *mdx* mouse muscle immunolabeled with anti-F4/80, a pan-macrophage marker (red). Note that some muscle fibers have been invaded and obliterated by large numbers of macrophages (white asterisk) while other, small regenerating fibers are surrounded but not invaded by macrophages (black asterisk). Bar = 50 μm. (B) Section of 4-week-old muscle immunolabeled with antibodies to F4/80 (red) and CD206 (green), a marker for M2-biased macrophages. Blue structures are nuclei binding DAPI reagent. This inflammatory lesion in dystrophic muscle contains proinflammatory, M1-biased macrophages (red) that can promote muscle damage, as well as antiinflammatory, M2-biased macrophages (orange), that can affect regeneration and fibrosis. Bar = 50 μm. [Reproduced, with permission, from reference (343).] (C) Characteristics of M1-biased and M2-biased macrophages in dystrophic muscle.

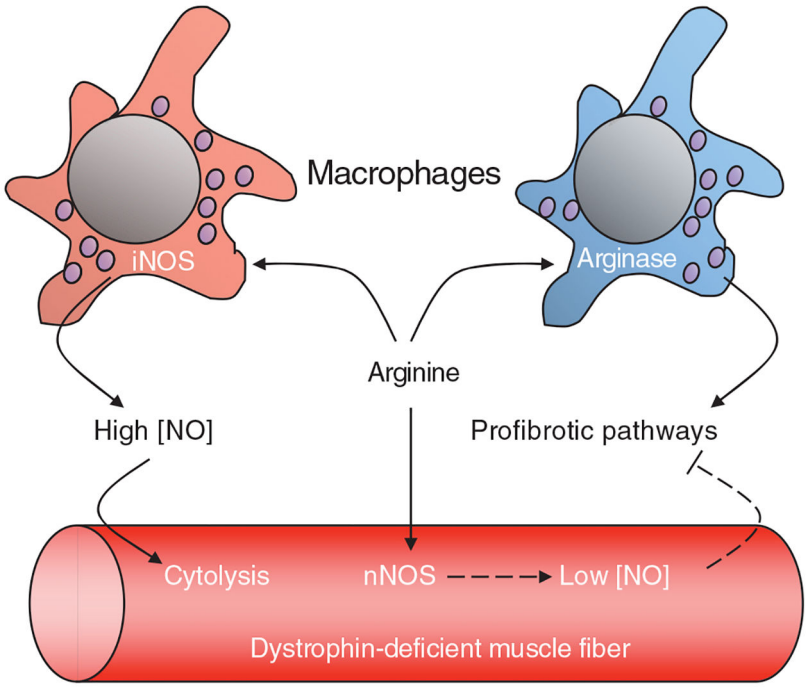



**Figure 6.**

Competition for arginine in *mdx* muscles can affect the pathology of muscular dystrophy. Because arginine is a conditionally essential amino acid in injured and diseased tissues, enzymes that metabolize arginine can compete for substrate. Normally in injured muscle, iNOS and arginase in macrophages and nNOS in muscle fibers compete for arginine. Muscle nNOS transcription is greatly reduced as a consequence of dystrophin deficiency, which increases arginine availability for iNOS and arginase. This amplifies muscle pathology because of muscle fiber lysis by iNOS-mediated mechanisms and muscle fibrosis mediated by arginase-mediated mechanisms. [Adapted, with permission, from reference (352).]

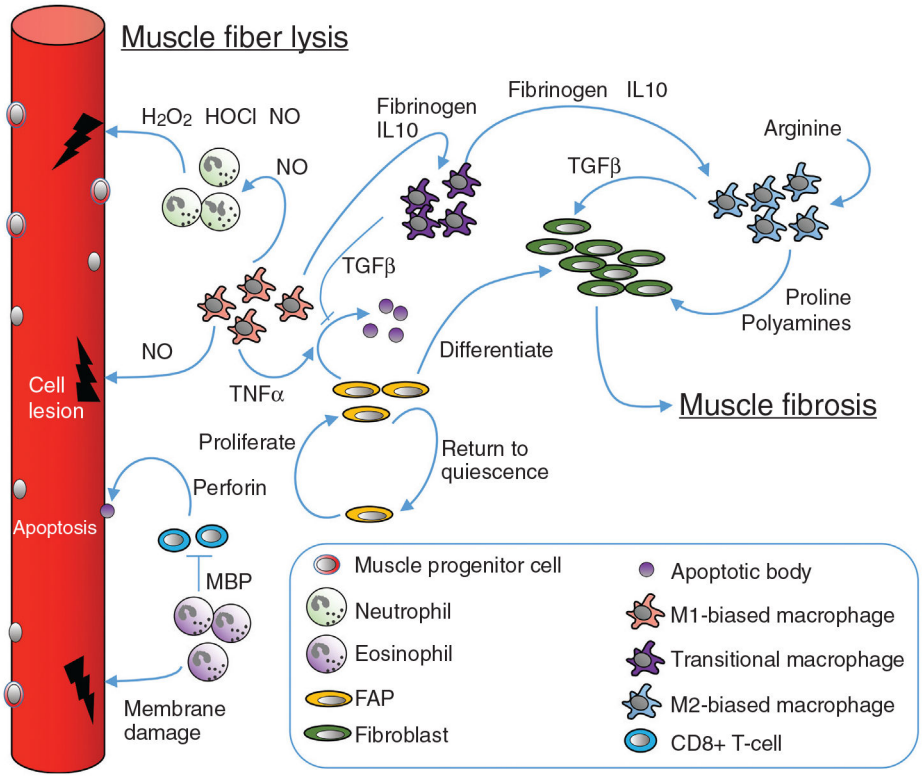
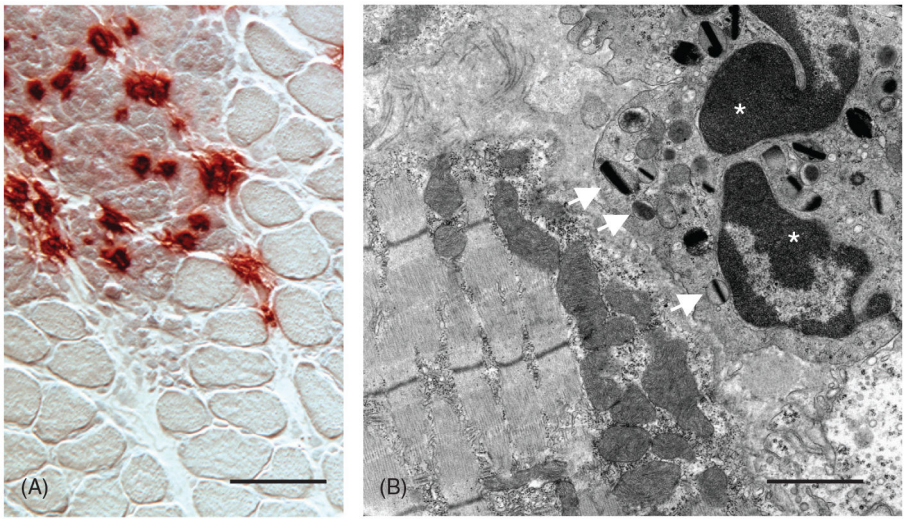



**Figure 7.**

Chronic muscle damage in dystrophinopathies causes dysregulation of the immune response that is adapted to acute injuries. Chronic long-term damage and inflammation of dystrophic muscle can amplify muscle fiber damage and fibrosis. Macrophages increase cytolysis of dystrophic muscle fibers by the release of high levels of NO. Macrophage-derived NO can also amplify neutrophil-mediated damage to muscle fibers, which can lyse muscle by producing free radicals including superoxide, hydrogen peroxide ($H_2O_2$), hypochlorous acid (HOCl), nitric oxide (NO), and peroxynitrite. CD8+ cytotoxic T-lymphocytes also contribute to muscle pathology by inducing myonuclear apoptosis through perforin-mediated mechanisms. The cytotoxicity of CD8+ cells can be diminished by eosinophil MBP, although eosinophils also amplify muscle fiber damage through MBP-independent mechanisms. M1-biased macrophages in dystrophic muscle are driven to an M2-biased phenotype by IL10 and fibrinogen which can subsequently increase muscle fibrosis by providing substrate to fibroblasts for connective tissue production through arginase-dependent events and by the release of TGFβ, which increases connective tissue production by fibroblasts.



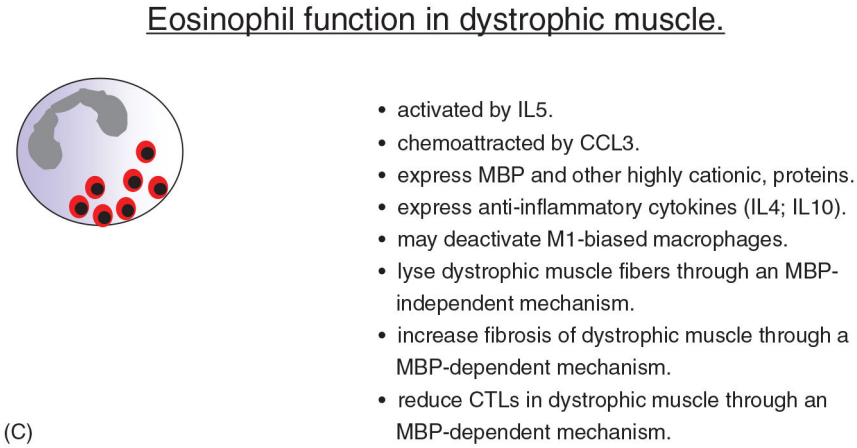




**Figure 8.**

Eosinophils in dystrophinopathy. (A) Inflammatory lesion in 4-week-old *mdx* mouse muscle in section stained with anti-MBP to indicate locations of MBP-expressing eosinophils (red). Note the elevated numbers of eosinophils in areas of increased connective tissue accumulation between muscle fibers. Also, note that the cytoplasmic organization of muscle fibers is disrupted in areas enriched with eosinophils, indicating fiber damage, but smooth in areas lacking eosinophils, indicating healthy fibers. Bar = 50 μm. (B) Electron micrograph of a portion of a muscle fiber in 12-month-old *mdx* mouse (left) in close apposition to an eosinophil with a multilobed nucleus (white asterisks). At the pole of the eosinophil closest to the muscle fiber, vesicles containing rods of MBP and other cationic proteins (white arrows) are accumulated. Bar = 1.5 μm. [Reproduced, with permission, from reference (357).] (C) Eosinophil function in dystrophic muscle.

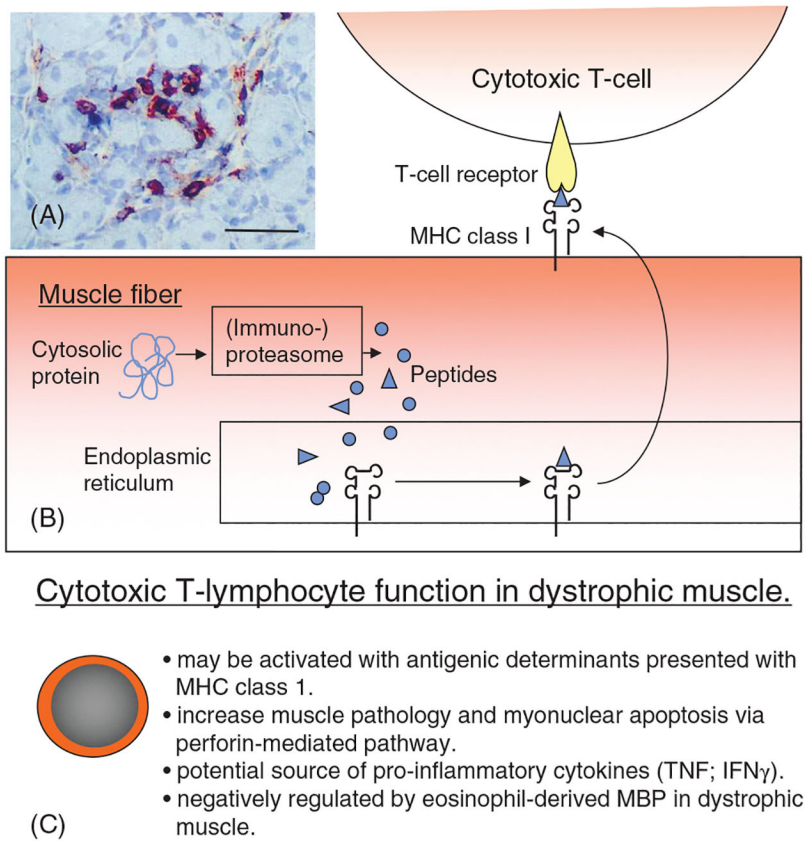



**Figure 9.**

Cytotoxic T-lymphocytes in dystrophinopathy. (A) Section of 4-week-old *mdx* muscle labeled with antibodies to CD8. Elevated numbers of CD8+ CTLs are present in inflammatory foci in dystrophic muscle. Bar = 50 μm. [Reproduced, with permission, from reference (298).] (B) Schematic of process leading to antigen presentation by muscle fibers leading to activation of CTLs. (C) CTL function in dystrophic muscle.

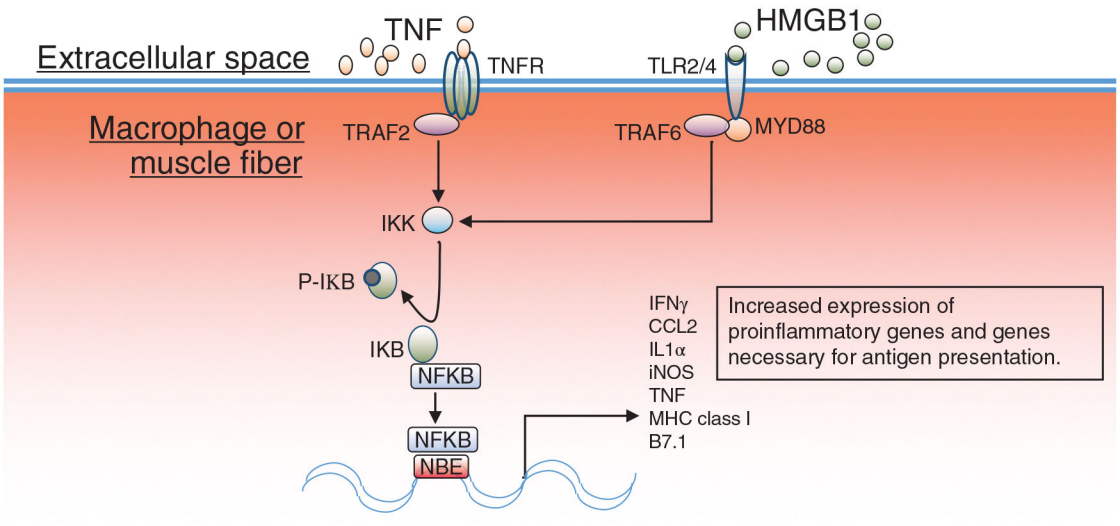



**Figure 10.**

Activation of the expression of proinflammatory molecules and molecules necessary for antigen presentation by MHC class 1. Both muscle fibers and macrophages can be activated to increase expression of proinflammatory molecules and MHC class 1 via NFκB activation. IκB kinase (IKK) can be activated by TNF binding to its receptor and acting through the adapter protein TRAF2 or by HMGB1 binding its receptor and acting through adapters TRAF6 or MYD88. Activated IKK then phosphorylates IκB, leading to its dissociation from NFκB, causing NFκB activation. Activated NFκB then translocate to the nucleus to bind to the NFκB response element (NRE) of target genes, to activate their expression.

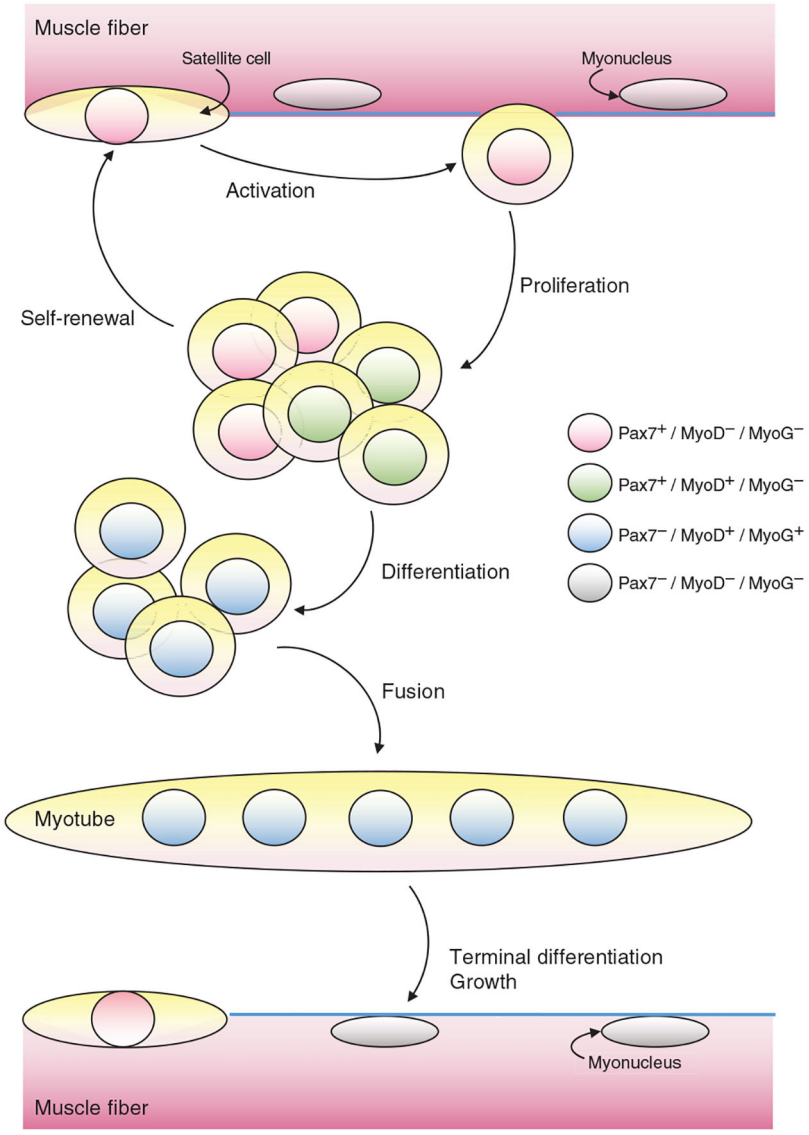



**Figure 11.**

Activation of myogenic precursor cells is essential for repeated muscle regeneration. Satellite cells are myogenic cells that reside in a quiescent state on the surface of muscle fibers and can be identified by their location and by their pattern of expression of myogenic transcription factors ($Pax7^+/MyoD^-/MyoG^-$). Following their activation by injury or disease, they enter the cell cycle to give rise to two daughter cells with the same developmental destiny or cells with nonidentical developmental paths. Proliferative daughter cells begin to express MyoD and continue to differentiate while other daughter cells return to the quiescent state and remain $Pax7^+/MyoD^-$. As the $Pax7^+/MyoD^+$ cells continue to differentiate, they permanently downregulate Pax7 and begin to express the transcription factor myogenin (MyoG) which is required for further differentiation. The $Pax7^-/MyoD^+/MyoG^+$ cells can fuse with other myogenic cells to form multinucleated myotubes. Myotubes then grow and begin to express genes required for terminal differentiation and become nascent muscle fibers. Eventually, nuclei derived from the originally activated

satellite cell population become myonuclei that reside within the muscle fiber, in which myogenic regulatory genes are permanently silenced ($Pax7^{-}/MyoD^{-}/MyoG^{-}$). [Adapted, with permission, from reference (316).]

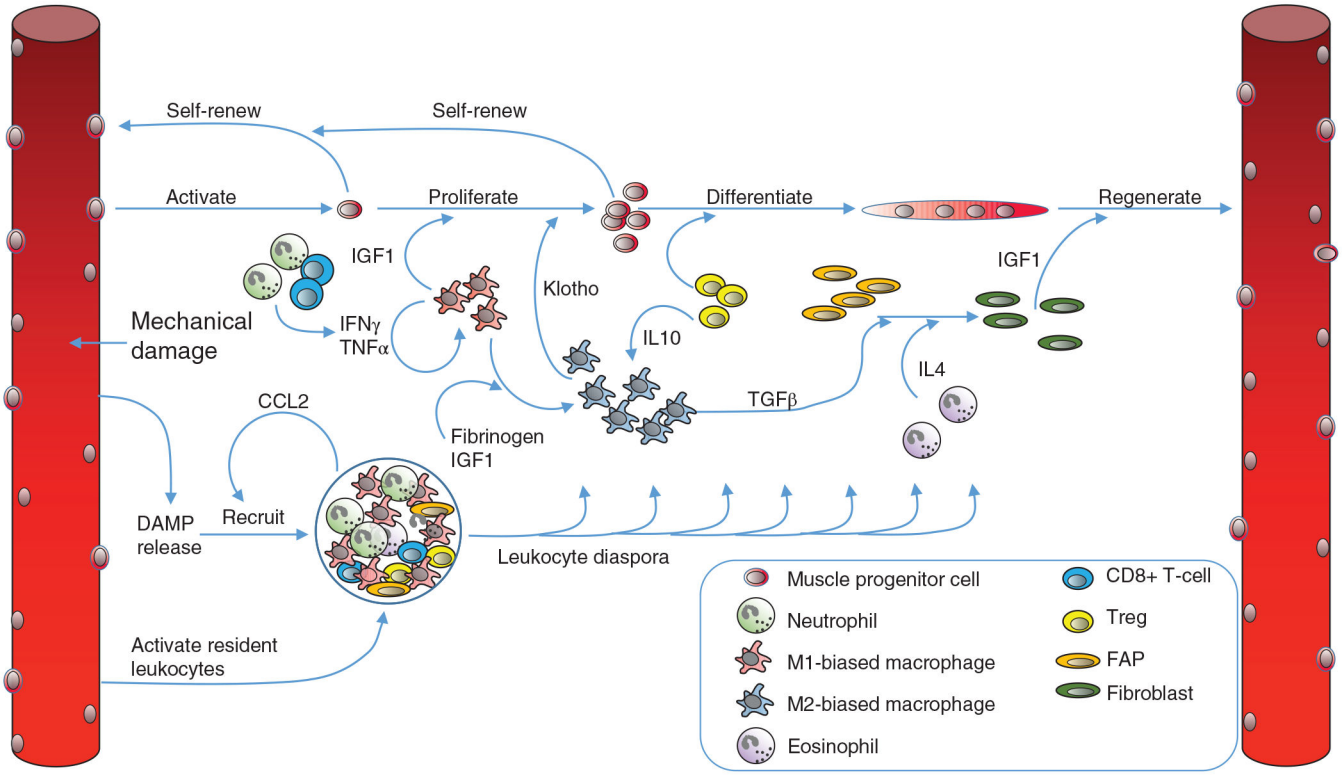



**Figure 12.**

Inflammation and regeneration are coupled in dystrophic muscle. Damage of dystrophic muscle fibers leads to the release of DAMPs that can recruit and activate leukocytes from the vasculature as well as leukocytes that reside in the muscle. Those leukocytes can further promote recruitment by the release of chemoattractants, such as CCLs. The activated leukocytes then disperse throughout the muscle where they can influence regeneration, in addition to promoting damage. Macrophages that dominate the initial inflammatory infiltrate can be biased toward an M1, cytolytic phenotype by Th1 cytokines such as TNF and IFNγ that can be released by neutrophils and CD8+ cells. In parallel with the recruitment and activation of immune cells, muscle fiber damage also activates satellite cells to begin their program of expansion and differentiation that contributes to muscle regeneration. M1-biased macrophages release IGF-1 that stimulates myogenic cell proliferation. M1-biased macrophages can also switch to an M2-biased phenotype following stimulation by fibrinogen or IGF-1 or by IL10 produced by Tregs. M2-biased macrophages can also contribute to the expansion of myogenic cell populations through the release of Klotho. M2-biased macrophages can also release TGFβ, that increases the differentiation of FAPs to become fibroblasts. Fibroblast production of IGF-1 promotes the growth and differentiation of myotubes to become muscle fibers.

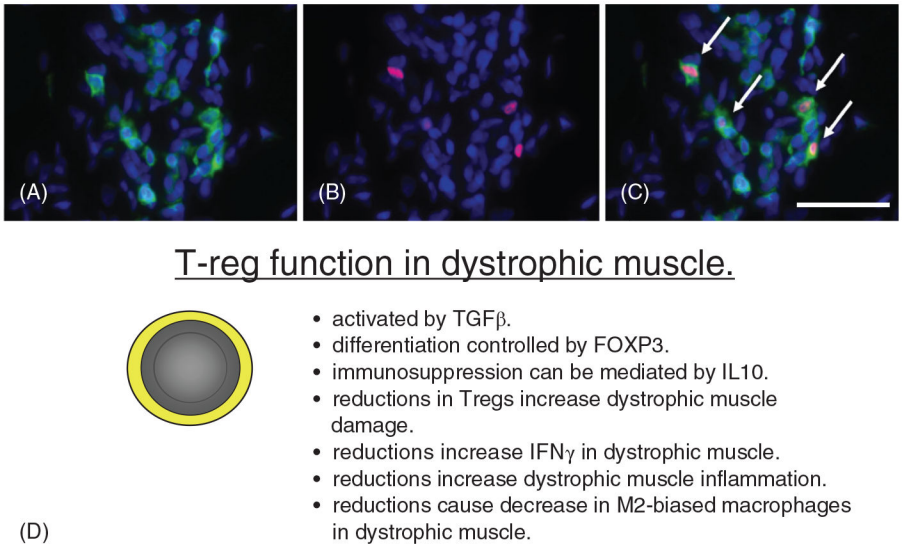



**Figure 13.**

Regulatory T-cells in muscular dystrophy. [(A)-(C)] Section of DMD muscle biopsy labeled with antibodies to CD3 (A; green) and FoxP3 (B; red) showing that a portion of CD3+ T-cells in DMD muscle are FoxP3-expressing Tregs (C; double-labeled, arrows). [Reproduced, with permission, from reference (344).] (D) Treg function in dystrophic muscle. Bar = 40 μm.

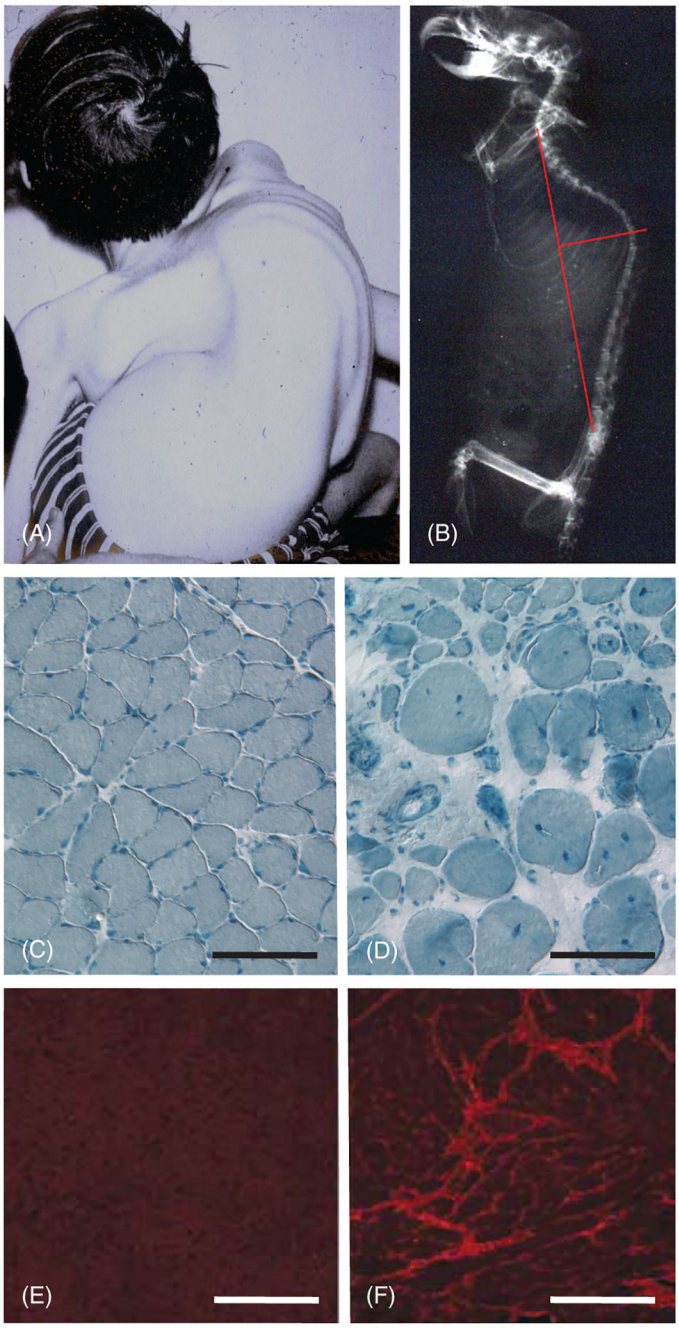



**Figure 14.**

Muscle and cardiac fibrosis are major features of the pathophysiology of dystrophinopathies. (A) Posterior view of thorax of DMD patient showing extreme kyphoscoliosis that is attributable, in part, to fibrosis of paravertebral muscles. (B) Radiograph of 18-month-old *mdx* mouse showing pathological curvature of the spine caused by increased fibrosis of paravertebral muscles. [Reproduced, with permission, from reference (352).] [(C) and (D)] Cross section of quadriceps muscles from 24-month-old wild-type (C) or *mdx* mouse (D) stained with hematoxylin. Fibers in wild-type muscle have similar size and are separated by little connective tissue although *mdx* muscles show large variability in fiber size and

extensive accumulation of connective tissue between fibers. Bars = 100 μm. [Reproduced, with permission, from reference (318).] [(E) and (F)] Section through myocardium of 1-year-old wild-type (E) and *mdx* (F) mice stained for collagen type 1 shows fibrous lesions that occur in *mdx* myocardia are absent in wild-type mice. Bars = 50 μm. [Reproduced, with permission, from reference (353).]

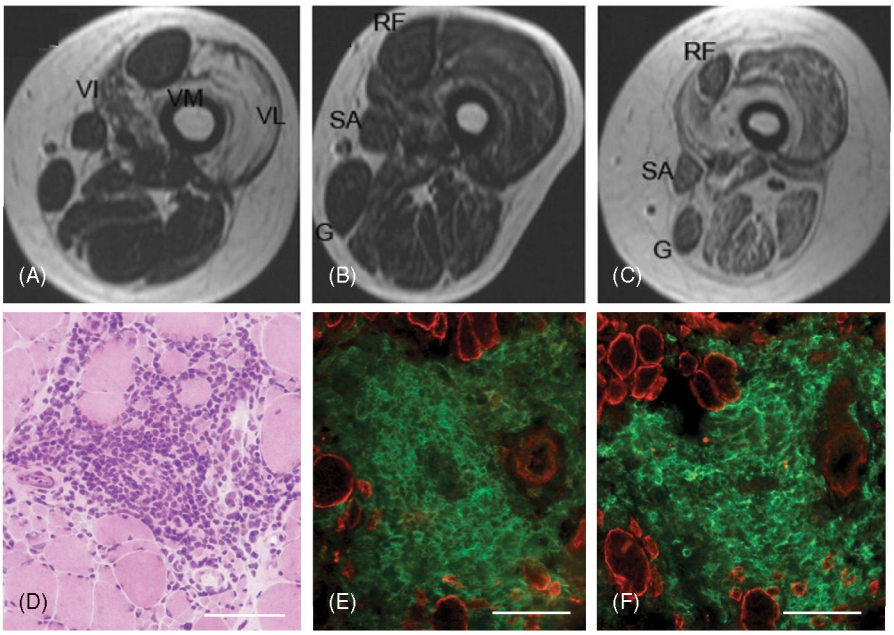



**Figure 15.**

Muscle wasting in LGMD1B can be accompanied by extensive immune cell involvement that indicates an acquired immune response. [(A)-(C)] T1-weighted magnetic resonance imaging of transverse sections of thighs of three LGMD1B patients. Muscles appearing dark in the image are increasingly replaced by fat and connective tissue, appearing white. Muscles affected include rectus femoris (RF), sartorius (SA), vastus medius (VM), vastus intermedius (VI), vastus lateralis (VL), and gracilis (G). Hematoxylin and eosin-stained section of biopsied muscle tissue from LGMD1B patient (D) shows greatly elevated numbers of inflammatory cells in lesions within the muscle. (E) Immunohistochemistry of sections of biopsied muscles labeled with antibodies to CD4 (green) and dystrophin (red) shows that many of the leukocytes in inflammatory foci are T-cells. (F) Immunohistochemistry of sections of biopsied muscles labeled with antibodies to CD20 (green) and dystrophin (red) also shows elevated numbers of B-cells in the lesions. Bars = 70 μm. [Reproduced, with permission, from reference (175).]

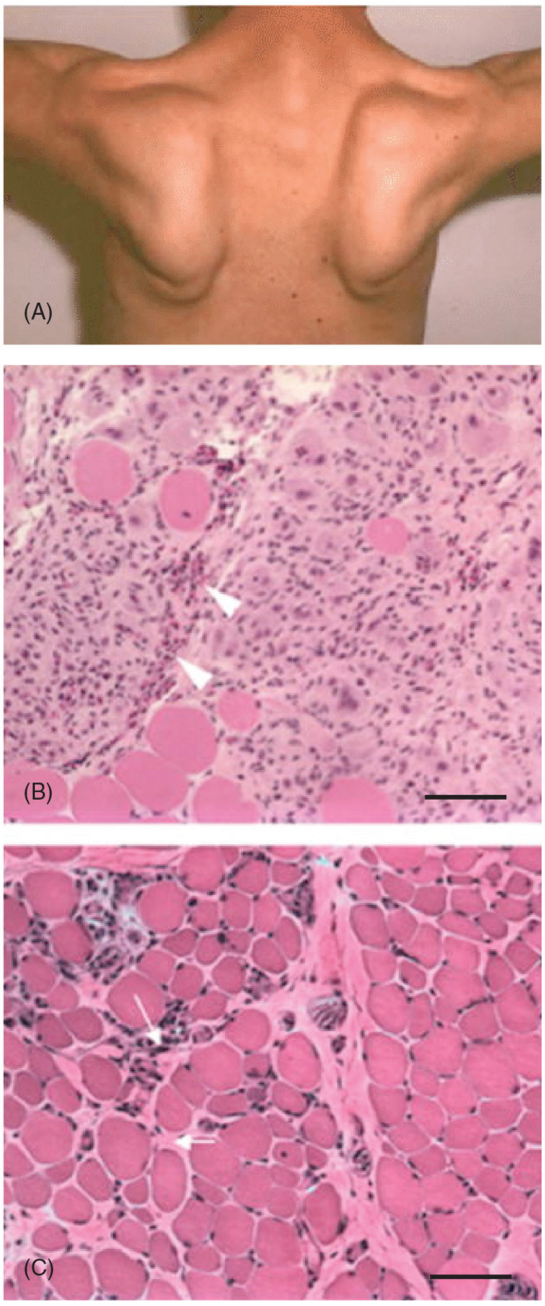



**Figure 16.**

Muscle wasting and inflammation in LGMD2A. (A) LGMD2A is frequently associated with "winged scapulae" attributable to weakening and loss of muscles of the shoulder girdle. [Reproduced, with permission, from reference (7).] (B) Muscle histology shows that muscle fibers in LGMD2A can be replaced by vast numbers of inflammatory cells, that include elevated numbers of eosinophils (arrowheads) in some cases. (C) Muscle histopathology also includes increased deposition of fatty tissue (arrows) and thickening of perimysial and endomysial connective tissue. Bars = 80 μm. [Reproduced, with permission, from reference (276).]

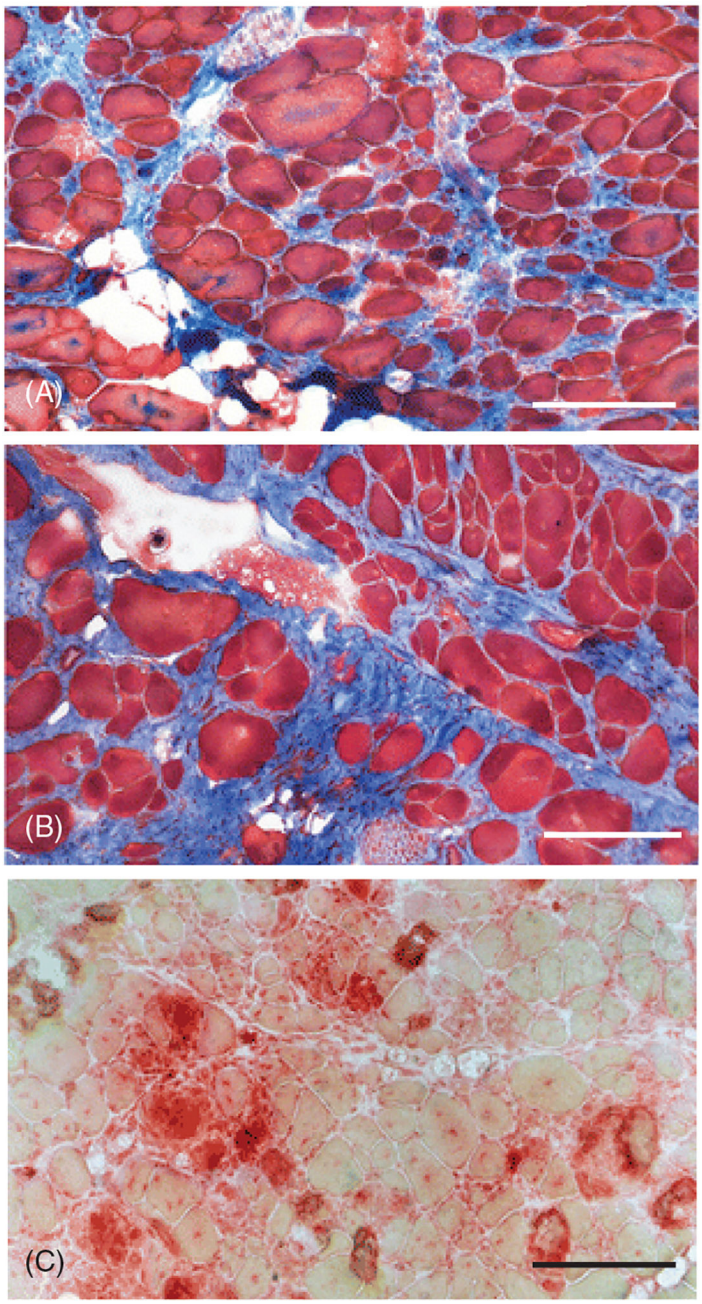



**Figure 17.**

β-sarcoglycan null mice, a model for LGMD2E, experience extensive muscle fibrosis and inflammation. [(A) and (B)] One-year old, βSCG mice show large accumulations of connective tissue (blue) between muscle fibers in Masson's trichrome-stained sections of quadriceps muscles (A) or diaphragm (B). Elevated numbers of macrophages (C), which appear red in acid phosphatase-stained sections, occur in elevated numbers in the interstitial space between muscle fibers. Bar = 100 μm. [Reproduced, with permission, from reference (108).]

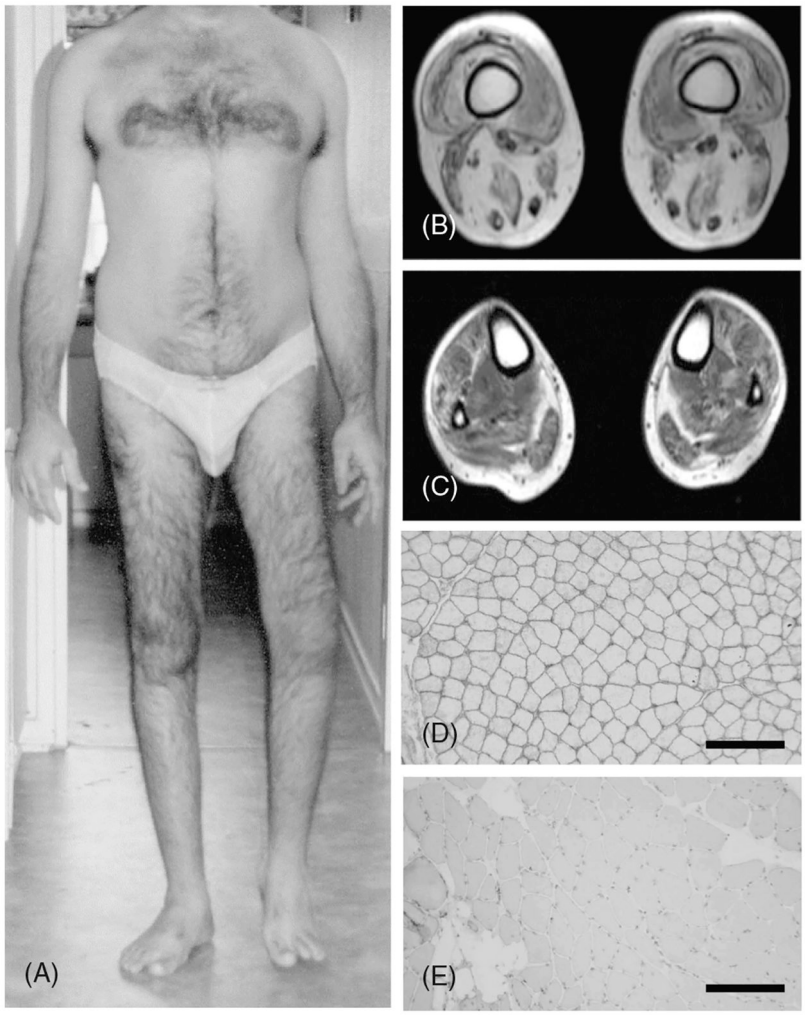



**Figure 18.**

Characteristics of LGMD2B. (A) LGMD2B patient showing wasting of proximal and distal, lower limb musculature. The patient also shows characteristic walking patterns that include reduction of normal heel strike during foot placement. [(Reproduced, with permission, from reference (200).] [(B) and (C)] T1-weighted MRI of thigh (B) and calf (C) of LGMD2B patient showing extensive replacement of musculature (dark grey) with fat and connective tissue (white). [(Reproduced, with permission, from reference (8).] [(D) and (E)] Anti-dysferlin staining of healthy, human muscle biopsy (D) and LGMD2B muscle biopsy (E). The normal distribution of dysferlin at the muscle fiber surface is absent in LGMD2B muscles. Bar = 150 μm. [Reproduced, with permission, from reference (172).]

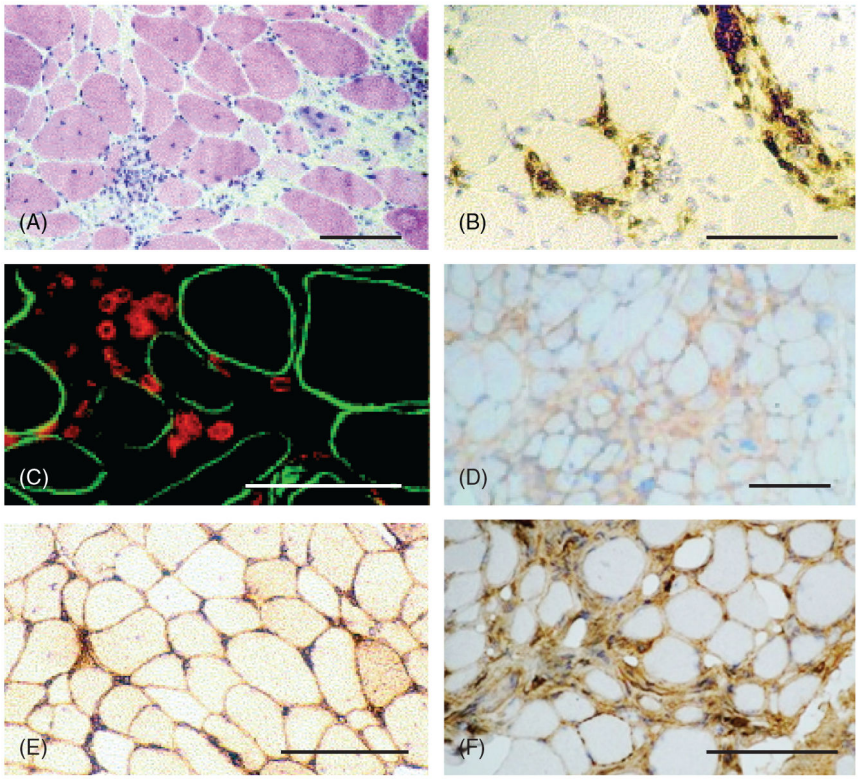



**Figure 19.**

Immune cell infiltrates in LGMD2B muscle. (A) Hematoxylin and eosin-stained muscle section of LGMD2B biopsy shows large interstitial spaces between muscle fibers that are occupied by inflammatory cells (dark blue) and fibrotic connective tissue. Bar = 100 μm. [Reproduced, with permission, from reference (62).] (B) Anti-CD3 staining (dark brown) shows that many of the immune cells in LGMD2B muscles are T-cells. Bar = 100 μm. [Reproduced, with permission, from reference (62).] (C) Indirect immunofluorescence antibody labeling of dystrophin (green) and CD8+ T-cells in LGMD2B muscle biopsy section show CTLs near or on surface of muscle fibers. Bar = 50 μm. [Reproduced, with permission, from reference (62).] (D) Anti-CD68 staining (brown) shows that many of the immune cells in inflammatory lesions in LGMD2B muscles are macrophages. Bar = 100 μm. [(Reproduced, with permission, from reference (372).] (E) Anti-MHC class 1 staining of LGMD2B muscle biopsies shows localization at the surfaces of muscle fibers suggesting antigen presentation by muscle fibers. Bar = 100 μm. [Reproduced, with permission, from reference (62).] (F) Section of LGMD2B muscle biopsy labeled with antibodies to C5b (dark brown) indicates complement activation occurs in dysferlin-deficient muscles. Bar = 100 μm. [Reproduced, with permission, from reference (372).]

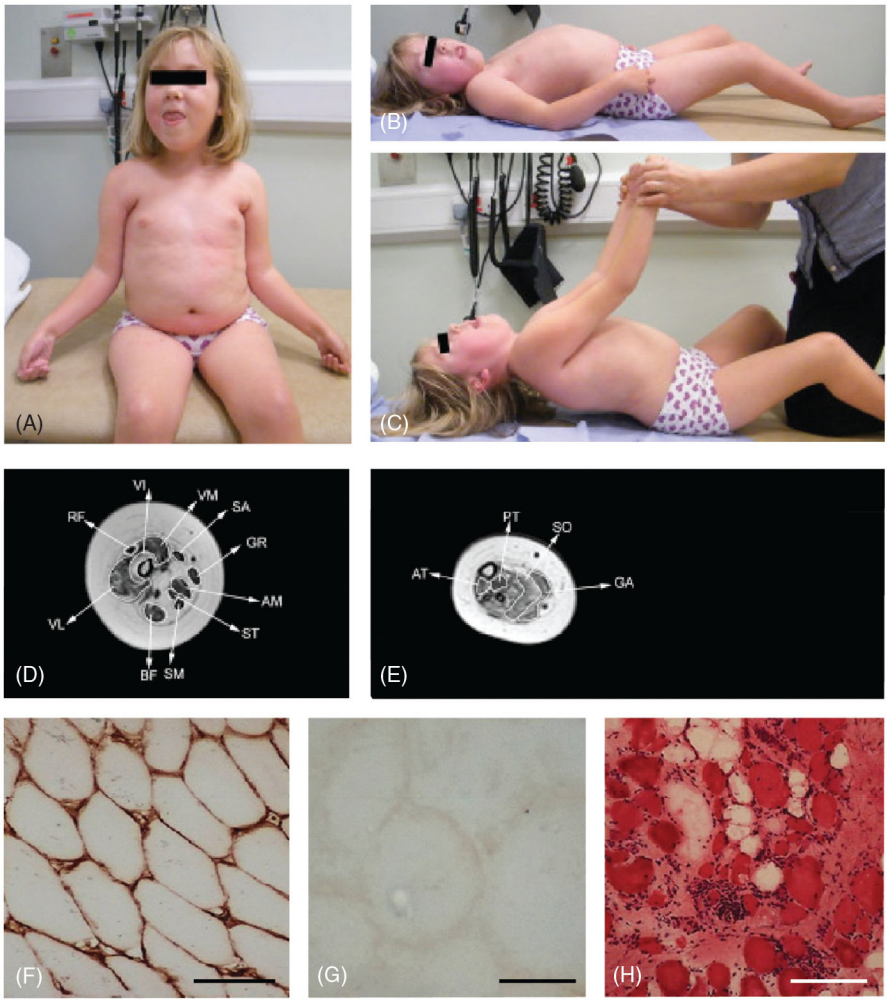



**Figure 20.**

Characteristics of MDC1A. (A) MDC1A patient with characteristic weakness of facial muscles and elbow flexions resulting from increased fibrosis. (B) Increased lumbar lordosis and knee flexions are also attributable to fibrotic contractures. (C) Weakness of neck musculature in MDC1A patient is exhibited when the patient is pulled up from a supine position. [Reproduced, with permission, from reference (105).] [(D) and (E)] T-weighted MRI of thigh (D) and leg (E) of 1-year-old MDC1A patient showing extensive muscle atrophy, fatty infiltration, and edema of lower limb musculature. PT, posterior tibial; SO, soleus; GA, gastrocnemius; AT, anterior tibial; VI, vastus internus; VM, vastus medialis; SA, sartorius; GR, gracilis; AM, adductor magnus; ST, semitendinosus; SM, semimembranosus; BF, biceps femoris; VL, vastus lateralis; RF, rectus femoris. [Reproduced, with permission, from reference (195).] (F) Anti-merosin immunolabeling of a section of biopsied muscle from healthy subject shows each muscle fiber encircled by a continuous layer of merosin in the basal lamina. Bar = 70 mm. [Reproduced, with permission, from reference (176).] (G) Anti-merosin labeling of a section of biopsied muscle from an MDC1A shows complete absence of merosin in the basal lamina. Bar = 30 mm. [Reproduced, with permission, from reference (176).] (H) Hematoxylin and eosin-stained muscle section of MDC1A biopsy

shows large interstitial spaces between muscle fibers (dark red) that are occupied by fat (pale spheres) inflammatory cells (dark blue) and fibrotic connective tissue. Bar = 100 mm. [Reproduced, with permission, from reference (176).]

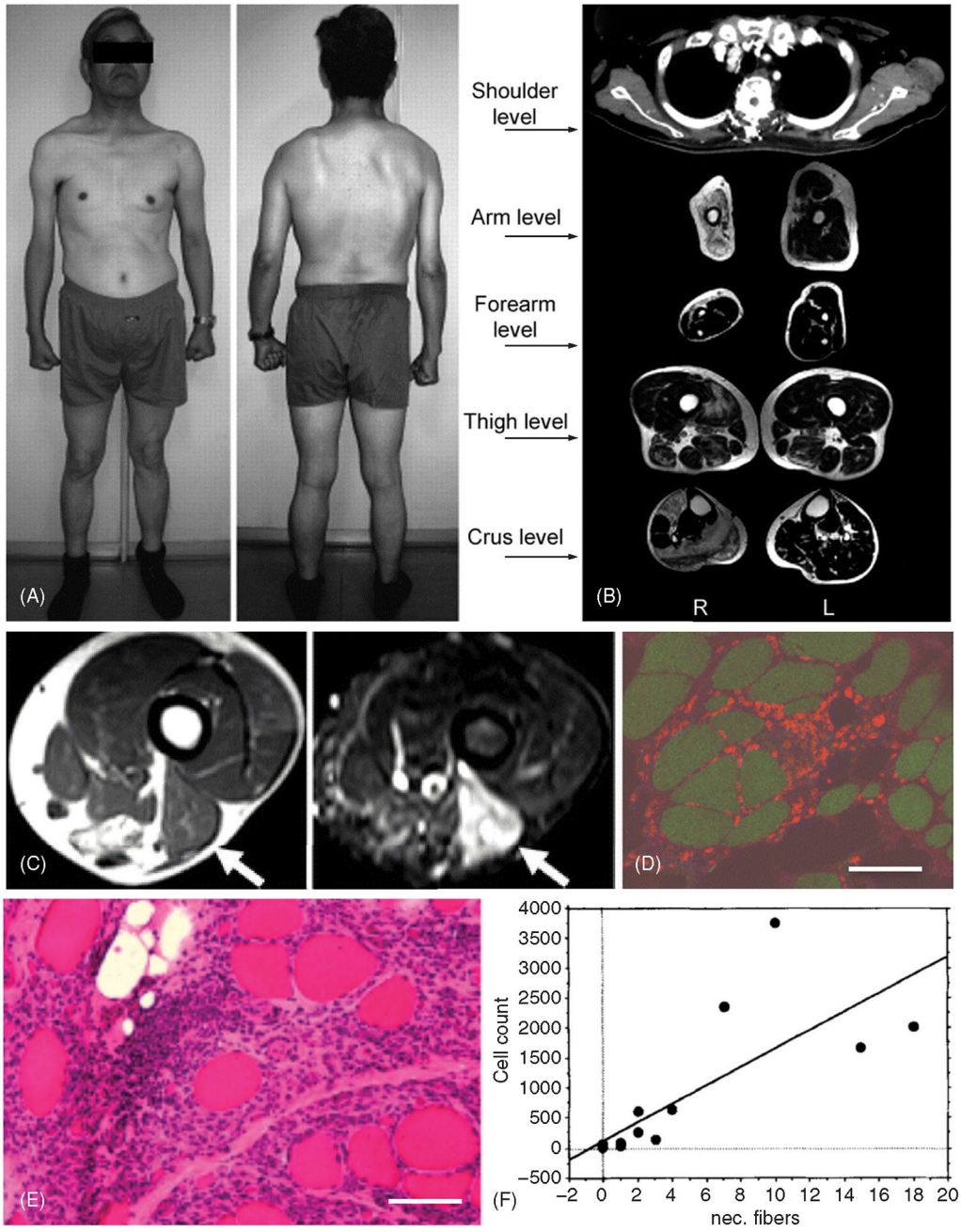



**Figure 21.**

Effects of FSHD on muscle structure and inflammation. (A) FSHD patient showing asymmetric muscle wasting on right side of body, including muscles of shoulder girdle, humeral, and lower limb muscle. (B) T2-weighted MRI images of right and left, upper, and lower limbs of same patient showing extensive muscle loss and replacement by fat, that is particularly prominent in arm level and crus level images. [Reproduced, with permission, from reference (304).] (C) T1-weighted (left) and T2-weighted (right) MRI of FSHD patient. Arrow indicates site of muscle showing hyperintensity in T2 image, that was sampled by biopsy, and found to contain highly elevated numbers of leukocytes. (D) Section

of muscle biopsy that is shown in (C), that was immunolabeled with antibodies to CD8 shows large numbers of CD8+ CTLs (red) surrounding muscle fibers (green). Bar = 50 μm. [Reproduced, with permission, from reference (101).] (E) Muscle biopsy from FSHD patient stained with hematoxylin and eosin shows extensive inflammation and fibrosis in the interstitium between muscle fibers. Bar = 70 μm. [Reproduced, with permission, from reference (58).] (F) Quantitative histological analysis of inflammation of muscle biopsies from FSHD patients shows a positive correlation between the number of inflammatory cells ("cell count") and the number of necrotic fibers ("nec. fibers"). [Reproduced, with permission, from reference (11).]