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Immune mediated mechanisms potentially regulate the disease time course of Duchenne muscular dystrophy and provide targets for therapeutic intervention

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Abstract

Duchenne muscular dystrophy is a lethal muscle wasting disease that affects boys. Mutations in the dystrophin gene result in the absence of the dystrophin glycoprotein complex (DGC) from muscle plasma membranes. In healthy muscle fibers, the DGC forms a link between the extracellular matrix and the cytoskeleton to protect against contraction-induced membrane lesions and to regulate cell signaling. The absence of the DGC results in aberrant regulation of inflammatory signaling cascades. Inflammation is a key pathological characteristic of dystrophic muscle lesion formation, but the role and regulation of this process in the disease time course has not been sufficiently examined. The transcription factor, NF- κ B has been shown to contribute to the disease process and is likely involved with increased inflammatory gene expression, including cytokines and chemokines, seen in dystrophic muscle. These aberrant signaling processes may regulate the early time course of inflammatory events that contribute to disease onset. This review critically evaluates the possibility that dystrophic muscle lesions in both DMD patients and *mdx* mice are the result of immune-mediated mechanisms that are regulated by inflammatory signaling and also highlights new therapeutic directions.

Introduction

Duchenne muscular dystrophy (DMD) is a lethal, human muscle wasting disease caused by mutations in the dystrophin gene that result in the loss of the dystrophin protein [1,2]. In normal muscle cells, the dystrophin-glycoprotein complex (DGC) (Figure 1) is localized to the sarcolemma forming a link between the extracellular matrix (ECM) and cytoskeleton [3]. This complex is thought to protect muscle cells from contraction-induced injury and preserve cell

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viability through both mechanical and signaling roles [4,5]. In DMD, dystrophin and the associated DGC proteins are absent from the sarcolemmal membrane resulting in altered mechanical and signaling functions which contribute to membrane fragility, necrosis, inflammation, and progressive muscle wasting [6].

Although mechanical injury and membrane defects are important factors promoting dystrophic disease pathology, neither fully explains DMD disease onset and progression [7,8]. Aberrant intracellular signaling cascades that regulate both inflammatory and immune processes contribute substantially to the degenerative process [9-11]. Up-regulated inflammatory gene expression (e.g., cytokines, chemokines, and major histocompatibility complexes) and activated immune cell infiltrates (e.g., T lymphocytes and macrophages) are evident during early disease stages in dystrophic muscle and play a critical role in muscle wasting [12-18]. The purposes of this review are (1) to propose and evaluate the possibility that the onset of dystrophic muscle lesions in both DMD patients and *mdx* mice are the result of immune-mediated mechanisms, (2) to explore the roles of NF- κ B, cytokines, and chemokines as regulators of these immune mechanisms and, (3) to highlight therapeutic options to blunt these processes,

Immune mediated dystrophic muscle lesion formation

An understudied area to delineate the time course of DMD pathology during early disease onset is the contribution of immune mechanisms. Time course refers to the characteristics of dystrophic pathology that arise over time from pre onset through early progression of the disease. One advantage of this knowledge is that it can reveal significant information to guide treatment at a specific disease stage. For example, immune cell infiltration in dystrophic muscle was once thought to be a non-specific reaction to damaged muscle fibers [19-21], but is now considered to be a specific response that directly contributes to muscle lesion formation [13, 14,22-24]. Because immune cell infiltration (i.e., neutrophils and macrophages) precedes initial disease onset in dystrophic muscle [12-14], it is not surprising that immunosuppressive therapies can significantly blunt muscle wasting when administered prior to disease onset [13,14,25-29]. Although comprehensive time course data sets are limited, those that are available suggest early immune cell infiltration in DMD patients and *mdx* mice represent an important, but underappreciated aspect of dystrophic muscle pathology.

Immune cells can contribute substantially to the initiation and progression of muscle pathology (Figure 2) [30,31]. In dystrophic muscle, macrophages and T-cells are the primary infiltrating immune cell types [13,14,19,20,32]. Macrophages, when triggered by inflammatory stimuli, lyse muscle fibers through the production of nitric oxide [14,24]. Macrophages may also engulf and then present antigens to T-cells to induce their activation; however, this process has not been characterized in dystrophic muscle [33]. CD8+ T-cells trigger muscle fiber death and CD4+ T-cells can contribute to this process by providing inflammatory stimuli (i.e., cytokines) to CD8+ cells and other immune cells [13,33]. The high concentrations of macrophages and T-cells observed in dystrophic muscle from early disease stages and persisting through later stages indicates the importance of these cells in the initiation and progression of dystrophic muscle lesions [13,14,19,20,32]. Additionally, mast cells, eosinophils, and neutrophils are believed to contribute to dystrophic muscle fiber wasting [25,34-37]. The role of these cells in dystrophic muscle lesion formation is less clear, but data indicates that they are significant factors in disease progression.

DMD Patients

In DMD patients, immune cells are present throughout perivascular, perimysial, and endomysial regions of skeletal muscle [20]. At age 2-8 years, the infiltrating mononuclear cells are predominantly macrophages (~38%) and T-cells (~62%), with minimal B-cell (~1%)

infiltration [19,20]. The infiltrating T-cell population is composed predominantly of CD4+ cells with some CD8+ cells [32]. Mast cells have also been implicated in the initiation and progression of muscle lesions in DMD. Peak mast cell infiltration occurs by age 3 years and there is a 10-fold increase in these cells in skeletal muscle from DMD patients when compared to healthy individuals [36].

Major Histocompatibility Complex (MHC) class I proteins are expressed on most nucleated cells, whereas MHC class II proteins are only expressed on professional antigen presenting cells (APCs) like macrophages, and B-cells. Infiltrating mononuclear cells are strongly reactive for both MHC class I and II proteins in DMD skeletal muscle [32]. In Patients as young as 1-year-old, MHC II proteins are consistently observed on infiltrating mononuclear cells indicating the presence of APC's in the muscle tissue from an early age [38]. Skeletal muscle fibers are one of a few cell types that have very little or no detectable MHC I proteins on their surface [32,38-41], so it is striking that muscle fibers from DMD patients have a ~3-fold increase of these proteins [38]. MHC I proteins are detectable on skeletal muscle fibers from these patients as early as age 6 months [32,38]. MHC I staining can be observed on morphologically normal muscle fibers, necrotic fibers, regenerating fibers and fibers invaded by inflammatory cells [32,38-41]. This striking pattern of MHC production in dystrophic muscle could indicate that immune effector mechanisms are targeting specific dystrophic muscle antigens and contributing to disease pathology. Specifically, T-cells may recognize antigens bound to the surface of MHC proteins located on muscle fiber cell membranes [19].

Evidence of polyclonal expansion of T-cells has been noted in DMD patients and suggests increased immunoreactivity, albeit to unknown antigens [22,23]. T-cells isolated from DMD patients contain V β 2 T-cell receptor (TCR) rearrangements [22,33]. The majority of these patients exhibit a conserved amino acid sequence in the highly variable region of the TCR believed to interact with antigen, which is not observed in muscle tissue from healthy individuals. In other inflammatory muscle diseases, such as polymyositis, there is a predominance of V β 15 rearrangements not the V β 2 rearrangements that occur in DMD. These data indicate that the TCR rearrangements observed in DMD patients are specific for this disease [23]. Collectively, these data indicate that APC's and T-cells are present early (e.g., age 6-12 months) and may contribute to disease pathogenesis by targeting unknown antigens present on dystrophic muscle fibers. However, there is still insufficient data that fully characterizes the immune response in DMD patients, and therefore, much of the understanding about the role of the immune system and its effects on dystrophic muscle have come from *mdx* mice, a reasonable but not complete animal model for DMD.

Mdx mice

Mdx mice have been used to study the muscle lesion formation that results from immune cell infiltration because the immune cell populations in their muscles resemble those seen in DMD patients [13,14,19,20,32,42]. In DMD patients and *mdx* mice, muscle lesion formation is associated with immune cell infiltration that is clearly different from non-specific inflammatory responses to muscle injury [13,14,30,34-36]. The primary immune cells evident in skeletal muscle of *mdx* mice are macrophages, T-cells and neutrophils; however, other inflammatory cells are also believed to contribute to the observed muscle pathology (Table 1). In *mdx* mice, immune cell infiltration is an early pathological event that occurs between age 2-4 weeks and then decreases in severity by age 3 months [42].

Macrophage infiltration is the most prominent immune feature observed in *mdx* muscles. The concentration of these cells are elevated in *mdx* muscles as early as age 2 weeks and peak between ages 4 to 8 weeks [12-14,42]. Macrophages have a variety of important immunoregulatory and inflammatory functions that are not fully understood. Recently, two subpopulations of macrophages have been identified in *mdx* muscle tissue that may influence

muscle degeneration and regeneration depending on the proportion of these cells present [24]. The M1 population are pro-inflammatory and promote muscle cell lysis through inducible nitric oxide synthase (iNOS) dependent mechanisms while the M2 population enhance muscle regeneration by inducing satellite cell proliferation [24]. Cytokines play an important role in the regulation of the two populations of macrophages present in dystrophic muscle [24]. TNF α and IFN γ stimulate the expression of iNOS in M1 macrophages while IL-4 and IL-10 decrease iNOS expression and promote M2 activation [24]. IL-10 activated M2 macrophages are thought to promote satellite cell proliferation and muscle regeneration [24]. Although there is a population of macrophages in dystrophic muscle that supports regeneration, antibody-mediated depletion of macrophages in *mdx* mice, beginning at age 6 days and continuing to age 4 weeks, results in a >75% reduction of injured muscle fibers, suggesting an important role of these cells in the development of muscle lesions [14]. Future research might focus on ways to block the production of cytokines promoting the M1 phenotype and shift the balance of these cells to the M2 phenotype to dramatically improve the regenerative process.

Elevated concentrations of activated CD8+ and helper CD4+ T-cells are present in affected muscles of *mdx* mice between age 4-8 weeks, but rapidly decrease in concentration by age 14 weeks [13,42]. Activated CD4+ (18-fold) and CD8+ (17-fold) cells are significantly elevated in skeletal muscle from *mdx* mice compared to WT mice. No increases of these cells have been observed in axillary or inguinal lymph nodes of *mdx* mice, suggesting that their activation is occurring in the muscle tissue, and not systemically [13]. T-cells may be involved in the pathogenesis of DMD through three mechanisms that promote cytotoxicity: perforin-induced killing, fatty acid synthetase [fas]-induced killing and cytokine-induced killing [13,33]. Fas-induced killing has been ruled out as a main contributor to dystrophic muscle fiber death; however, both perforin- and cytokine-induced killing are likely to be significant contributors [13,33,42].

Antibody-mediated depletion of CD8+ or CD4+ cells in *mdx* mice, beginning at age 6 days and continuing to age 4 weeks, results in a 75% and 61% reduction in muscle histopathology, respectively [13]. This positive outcome suggests an important role for these cells in the development of muscle lesions.

Clusters of neutrophils are observed in the muscles of *mdx* mice as early as age 2 weeks [12]. Muscle injury and membrane lysis can be caused by superoxide production mediated by infiltrating neutrophils [30]. Antibody-mediated depletion of neutrophils starting at age 19 days significantly reduces muscle necrosis (age ~21 days) and subsequent regeneration (age ~28 days) [25]. For example, ~17% of the tibialis anterior (TA) muscle is necrotic in *mdx* mice during initial disease onset, while neutrophil depleted age-matched *mdx* mice have less than 3% muscle necrosis [25]. This reduction in the early necrotic process indicates the importance of neutrophils as key regulators of the dystrophic disease time course and may provide insight into mechanisms of immunopathogenesis since they are one of the earliest infiltrating cell types.

Eosinophil concentrations in quadriceps muscles of *mdx* mice age 4 weeks are elevated (~20-fold) compared to WT controls [34]. By 30 to 32 weeks, the concentration in *mdx* quadriceps significantly decreases, but is still significantly elevated compared to age matched WT controls (~7-fold) [34]. In diaphragm muscles of *mdx* mice age 4 weeks, the concentration of eosinophils is only marginally elevated, but then the concentration significantly increases by 30 to 32 weeks when compared to WT control [34]. The time course of eosinophil infiltration in each muscle appears to parallel the overall muscle pathology time course and may indicate that these cells have an unknown function in muscle wasting [34]. Electron micrographs of *mdx* hindlimb muscles show that eosinophils infiltrate the basement membrane of muscle fibers and are found in close proximity to the surface of necrotic fibers. Eosinophils contain vesicles with rod-like inclusions, consisting of major basic protein (MBP), which are oriented parallel to the surface

of non-pathological muscle fibers. However, eosinophils lying next to pathological muscle fibers have MBP rods oriented perpendicular to muscle fibers and appear to protrude through fiber membranes [34]. MBP and other cationic proteins released by eosinophils form pores in target cell membranes that result in cell swelling, Ca^{2+} influx, and the leakage of cytosolic proteins [43,44].

Mast cells have been implicated in dystrophic disease processes in *mdx* mice and in DMD patients [35,36]. Mast cells contain granules filled with biogenic amines (e.g., histamine), proteoglycans, cytokines, and other enzymes, which are released upon stimulation [35,36]. Mast cell proliferation and degranulation are commonly observed in areas of grouped fiber necrosis [35,36]. Mast cell degranulation results in the release of proteases (chymase, tryptase, carboxypeptidase), which can induce membrane lysis of nearby cells and promote local areas of ischemia [36]. Additionally, the release of molecules like $\text{TNF}\alpha$ and histamine from mast cell granules can contribute to a pro-inflammatory environment that promotes muscle necrosis [28]. Treatment of *mdx* mice beginning prior to initial disease onset (age 19 days) with sodium cromoglycate (cromolyn), a blocker of mast cell degranulation, results in ~59% decrease in cumulative TA muscle damage by age 28 days [28]. Additional research of mast cells in dystrophic muscle may provide insight into mechanisms that regulate the microenvironment and ischemic conditions that result in fiber necrosis.

Muscle tissue from DMD patients and *mdx* mice is infiltrated with large populations of immune cells during the early stages of the disease [12-14,20,33,45]. In DMD patients and *mdx* mice, the high concentration of macrophages and T-cells and the low concentration of B-cells present in the muscle during initial disease onset indicate a cell-mediated immune response [13,14,19,20,32,38]. In *mdx* mice, elevated concentrations of macrophages and neutrophils are observed as early as age 2 weeks [12,14,22], while peak mast cell and eosinophil infiltration are observed during initial disease onset [34,35]. Remarkably, the dramatic increase in immune cell infiltration in the *mdx* mouse rapidly decreases after initial disease onset and may be one mechanism by which *mdx* mice are spared the debilitating pathology suffered by DMD patients [42]. Although many studies have reported elevated populations of immune cells the mechanisms of immune cell infiltration and the initiation of myonecrosis have not been fully characterized in either DMD patients or in *mdx* mice; this greatly limits our current understanding of these mechanisms and their regulation. However, because several studies demonstrated that depletion of inflammatory cells results in a dramatic improvement in dystrophic muscle pathology, it is likely that inflammation is a key contributor to muscle wasting. If true, then potential regulators of dystrophic muscle pathology could be cytokines and chemokines, which are known to increase immune cell activity and may directly trigger muscle cell death signals [11,17,18,46]. Additionally, upregulation of cytokines and chemokines in dystrophic muscle prior to disease onset could be due to changes in NF- κ B activity resulting from disrupted DGC signaling [9,47]. As noted below, there is reasonable evidence to support this regulatory scheme. A clear benefit of understanding the processes that regulate inflammation is that new therapeutic strategies to ameliorate DMD may emerge.

NF- κ B, cytokines, and chemokines in dystrophic muscle

Intracellular signaling pathways are chronically activated in dystrophic muscle due to the loss of intact DGC signaling, Ca^{2+} influx through stretch activated channels and the production of ROS [9,10,48-50]. In addition, it is well recognized that dystrophic muscle membranes are susceptible to contraction-induced injury; however mechanical stretch can also induce the activation of transcription factors like NF- κ B that regulate the balance of cell survival and cell death. NF- κ B has been shown to regulate the expression of many inflammatory genes, including cytokines and chemokines in both immune cells and muscle fibers [9,47,51,52]. Cytokine inflammatory mediators are secreted from infiltrating lymphocytes (e.g., T-cells),

myeloid cells (e.g., macrophages), and from damaged muscle fibers to induce further immune cell infiltration and activation [17,46]. Chemokines regulate T-cell differentiation, leukocyte extravasation, and chemotaxis of various immune cells, including macrophages and T-cells, to sites of tissue damage and inflammation [11,18]. The mechanisms that orchestrate immune cell infiltration and activation have not been identified [11,15-17], but evidence suggests these immune-mediated mechanisms could be regulated by cytokine and chemokine signaling in response to upregulated NF- κ B.

NF- κ B

NF- κ B encompasses the Rel family of transcription factors and regulates the expression of many inflammatory genes. Humans express five different Rel/NF- κ B proteins which can be separated into two different classes. The first class is synthesized as mature proteins and includes: RelA (p65), c-Rel and Rel-B. The second class must be proteolytically processed from large precursors to the mature proteins p50 and p52. RelA, c-Rel and RelB have nuclear localization signals while p50 and p52 do not. p52 and p50 must dimerize with either RelA, c-Rel or RelB to act as transcription factors. NF- κ B dimers containing RelA or c-Rel are sequestered to the cytoplasm by I-kappaB (I κ B) inhibitor proteins. Activation of NF- κ B is a ubiquitin-dependent process which requires the phosphorylation of I κ B inhibitor proteins by I κ B kinase (IKK) triggering the nuclear localization signal. NF- κ B is then free to enter the nucleus and activate gene expression for inflammatory mediators [53,54]. The DGC appears to be involved in the regulation of NF- κ B and in dystrophic muscle there is aberrant activation of this pathway during early disease stages (Figure 3). For example, NF- κ B /DNA binding activity is significantly higher in *mdx* diaphragm muscles at ages 15, 18, 30, and 60 days when compared to WT [9]. Increased NF- κ B activity during the early *mdx* disease time course coincides with and appears to be involved in cytokine and chemokine expression.

NF- κ B activity is increased in the diaphragm, TA, and gastrocnemius muscles of *mdx* mice [47]. In these muscles, NF- κ B is composed of the p50 and p65 subunits with p50 appearing to predominate in the TA and gastrocnemius [9,47]. Other NF- κ B subunits (c-Rel, Rel-B, and Bcl-3) do not contribute significantly to DNA binding activity [47]. Although total p65 levels are not increased in *mdx* muscles, the activated or phosphorylated (p-p65) levels are increased in immune cells and regenerating muscle fibers of *mdx* mice and also DMD patients [47]. Heterozygous deletion of the p65, but not the p50, subunit results in decreased muscle pathology and decreased macrophage infiltration.

Results of recent studies indicate that activation of p65 by IKK β is independent of classical I κ B degradation [47,55]. A conditional deletion of IKK β in myeloid cells decreases p-p65 in macrophages but not in regenerating muscle fibers. Deletion of IKK β in macrophages results in a 51% decrease in necrotic foci, a 58% decrease in TNF α , a 66% decrease in IL-1 β , and a 45% decrease in MCP-1 α . However, the reduction of pro-inflammatory cytokines does not reduce p-p65 in dystrophic myofibers indicating that additional factors contribute to NF- κ B activation in muscle fibers. When IKK β deletion is targeted for muscle, noticeable increases in regeneration occur; yet, no decreases in immune cells are observed. A reduction in TNF α is reported, but no significant change in expression of IL-1 β , MCP-1, RANTES, and MIP-1 α are apparent [47]. TNF α is believed to inhibit myogenesis by activating NF- κ B, which regulates MyoD expression [47,56]. IKK β -deficient *mdx* muscles have a 41% increase in MyoD immunostaining, indicating NF- κ B activity may hinder dystrophic muscle differentiation. *Mdx* mice treated with an IKK specific inhibitor have an 80% decrease in macrophage infiltration and 77% reduction of membrane lysis of muscle tissue, emphasizing the role of NF- κ B in the dystrophic disease process [47].

Cytokines and chemokines

Cytokine and chemokine expression appears to be entwined with the disease time course of dystrophic muscle in DMD patients and *mdx* mice (Figure 4) [9,11,16,17,57-59]. In DMD patients, expression of chemokines, including MCP-1, are up-regulated before age 2 years [16]. Elevated TNF α concentrations are observed in the serum of DMD patients; however, elevated expression of TNF α and IL-1 β is not consistently observed in muscle tissue [46,60, 61]. Overexpression of TGF- β is observed in DMD patients older than 6 months and likely contributes to loss of muscle function due to fibrotic scarring [16,57]. In *mdx* mice, pro-inflammatory cytokine and chemokine expression have been reported prior to onset of muscle degeneration and are believed to contribute to this process [9,12,13,17,18,42]. Elevated expression of MCP-1 is detectable at age 14 days and 112 days [11]. Increased TNF α and IL-1 β expression are detectable in *mdx* mice age 16 and 60 days. Muscle degeneration is followed by regeneration, fibrosis, and a decrease in immune cell infiltration [12,42,57-59]. TGF- β expression is elevated in *mdx* mice during the time following peak muscle denegation (>age 42 days) [58,59]. Increased expression of TGF- β is believed to contribute to a decrease in the pro-inflammatory immune response and the increased muscle fibrosis following disease onset [16,57-59]. Although there is evidence to support the role of cytokines and chemokines in the time course of dystrophic muscle wasting, the mechanisms have not been fully characterized.

MCP-1 is thought to be an important factor in skeletal muscle regeneration and macrophage chemotaxis. MCP-1 expression increases after muscle injury and promotes muscle repair. In the absence of MCP-1 muscle regeneration and strength recovery are impaired [62-64]. Macrophages respond to the release of MCP-1 after muscle injury and contribute to muscle regeneration and the removal of necrotic debris [62,65]. Satellite cells can induce macrophage chemotaxis through the release of MCP-1. Once macrophages arrive on site they amplify chemotaxis and muscle repair [66]. In dystrophic muscle, macrophages have been shown to significantly contribute to dystrophic muscle wasting [14]. Therefore, MCP-1 and macrophage infiltration in dystrophic muscle is likely an attempt at muscle repair; however, excessive macrophage infiltration results in additional muscle damage. The mechanisms that regulate macrophage infiltration and muscle damage have not been fully assessed, but may be linked with MCP-1 release.

IL-1 has been associated with inflammatory responses in muscle [67]. Both IL-1 α and IL-1 β have been identified as key contributors of inflammation in inflammatory myopathies [68, 69]. In muscle of patients with inflammatory myopathies, IL-1 α is localized to endothelial and inflammatory cells and IL-1 β is localized to inflammatory cells. The IL-1 receptors, IL-1RI and IL-1RII are localized to muscle fibers [68]. IL-1 is thought to have negative effects on muscle function, protein synthesis and metabolism [70]. Very low concentrations of IL-1 β , have been shown to inhibit insulin-like growth factor (IGF)-I stimulated protein synthesis and myoblast differentiation [71]. IGF-1 is an important component that regulates satellite cell recruitment to injured muscle and the resolution of inflammation [72], so IGF-1 regulation by IL-1 β may be very important in dystrophic muscle. Increases in IL-1 β expression have been noted in dystrophic muscle; however, its contribution to dystrophic muscle wasting has not been addressed.

TNF α is responsible for mediating many immune processes and has been prominently studied in connection with muscle pathology [73,74]. This cytokine is mostly synthesized by macrophages in response to antigens, but can also be produced by muscle cells. Skeletal muscle expresses TNF α receptors I (TNFR1) and II (TNFR2), which bind with TNF α to initiate intracellular signaling pathways. TNF α is known to inhibit contractile function of skeletal muscle, induce muscle wasting, and may be involved in nitric oxide (NO) production [74]. TNF α /TNFR1/NF- κ B signaling is believed to increase the production of reactive oxygen

species (ROS) via mitochondrial electron transport and increase activity of the ubiquitin/proteasome pathway [74]. ROS can also regulate TNF α /NF- κ B signaling, and thereby influence further catabolic processes, including protein loss, in mature muscle fibers [73].

TNF α -deficient *mdx* mice (*mdx*/TNF α ^{-/-}) have been used to determine the role of this cytokine in dystrophic muscle pathology [75]. *Mdx*/TNF α ^{-/-} mice age 4-8 weeks display no change in CK levels or muscle strength compared to *mdx* controls, although their body mass and muscle mass are significantly decreased by age 8 weeks. *Mdx*/TNF α ^{-/-} mice have significantly lower (~2-fold) pathological markers (macrophage and neutrophil invasion, hyaline fibers, and necrosis) in quadriceps muscles at age 8 weeks. In diaphragm muscles of these mice, pathological markers are significantly increased (~2-fold) at age 4 weeks, but not at age 8 weeks [75]. Pharmacological blockade of TNF α with a neutralizing antibody (Remicade), beginning at age 7 days, greatly reduces myofiber necrosis in TA muscles of *mdx* mice age 21 days when compared to both untreated *mdx* and *mdx*/TNF α ^{-/-} mice [25,29]. The reason for the differences in muscle pathology resulting from the two TNF α depletion techniques is unknown; however, TNF α appears to have a biphasic effect by which it can stimulate myogenesis in undifferentiated myocytes and increase catabolism in mature muscle fibers [73]. Therefore, one possibility is that treatments that deplete the concentration of TNF α , instead of eliminating gene expression, allow for low levels of TNF α to stimulate myogenesis without inducing muscle catabolism.

TGF- β 1 suppresses the immune system by preventing proliferation and function of specific T- and B-cell subsets by down regulating MHC I and II expression and inflammatory cytokine activity [57]. Elevated expression of TGF- β is associated with increased fibrosis in dystrophic tissue [58,76]. T-cells play an important role in mediating fibrosis in dystrophic muscle [77-79]. The TGF- β family of ligands and receptors are significantly elevated in the muscles of both DMD patients (age >6 months) and *mdx* mice (age 56 days); however they are not elevated in younger patients [16,57]. *Mdx* mice depleted of T-cells and/or B-cells have significantly less fibrosis and TGF- β 1 expression [78-80]. Antibody-mediated depletions of TGF- β 1 result in a significant reduction of connective tissue to near normal levels in diaphragm muscles of *mdx* mice age 12 weeks [59]. TGF- β 1 depletion also results in a significant increase in CD4⁺, but not CD8⁺ T-cells in *mdx* diaphragm muscles [59]. Although CD4⁺ cells concentration is increased, no difference in muscle degeneration is observed at age 12 weeks [59]. The increase in the concentration of CD4⁺ cells after the removal of TGF- β 1 indicates that this molecule may be an important suppressor of the immune response that contributes to dystrophic muscle wasting. The decrease in fibrosis and TGF- β 1 after the removal of lymphocytes indicates an important role for this molecule in the regulation of these cells. The induced expression of TGF- β following the initial disease onset phase may be one inhibitory mechanism attempting to suppress the immune response in dystrophic muscle, but may ultimately result in muscle fibrosis.

Increased activation of transcription factors, like NF- κ B, that regulate inflammatory genes are observed in dystrophic muscle which can ultimately result in the expression of cytokines and chemokines that regulate immune cell infiltration and activation [9,10,81]. Immune cells play a critical role in dystrophic muscle wasting [33]. In dystrophic muscle, chemokine expression is believed to be elevated before initial disease onset and likely initiates T lymphocyte and macrophage infiltration [11,16]. Increased expression of TNF α and IL-1 β before initial disease onset can trigger a pro-inflammatory immune response in dystrophic muscle. TNF α expression is associated with muscle wasting and is believed to be involved in dystrophic muscle pathology [25,29,75]. Expression of TGF- β is likely a mechanism that suppresses the pro-inflammatory immune response that occurs during disease onset, but results in fibrosis [16,57,58,59]. The role of chemokines and cytokines in the dystrophic disease time course has not been thoroughly investigated in DMD patients or *mdx* mice. Expression of these genes can be difficult to

measure because they are transiently expressed and not representative of the concentrations of released proteins. Although the role of these important regulatory molecules has not been thoroughly studied in DMD, they likely contribute substantially to the initiation and progression of this disease. Time course studies detailing cytokine and chemokine protein levels in *mdx* mice spanning before and after disease onset may enable the identification of potential time points at which anti-inflammatory therapies would provide the most beneficial results to blunt or prevent muscle wasting.

Emerging therapeutic options

In DMD patients and *mdx* mice, inflammation is a large component of muscle pathology [12-14,19,20,32,42]. Primarily macrophages and T cells are thought to contribute to muscle wasting in dystrophic muscle; however, as described above, other inflammatory cells are also significant contributors to this process [13,19,20,34-36,82]. Depletion or inhibition of these cells has been shown to significantly decrease dystrophic muscle pathology [25,28,34]. Although inflammation is a necessary part of the healing process, it can also contribute to the injury process [30,31]. Glucocorticoids have anti-inflammatory properties and have been used to treat DMD with some success; however, the side effects of these drugs often outweigh their benefit [26,83-88]. Numerous other anti-inflammatory therapies have been reported to improve healing, many of which may be useful in the treatment of DMD. Some of these emerging therapeutic options include stem cell treatment, anti-cytokine drugs, and inhibition of NF- κ B activity.

Stem cell therapies offer promising treatment options because of their ability to differentiate into different tissue types [89,90]. However, recent research has revealed that several types of stem cells have the ability to strongly suppress the immune response. Mesenchymal stem cells, hematopoietic stem cells, and placenta-derived multipotent stem cells exhibit immunosuppressive properties that are being studied for use in autoimmune diseases [91-94]. When these stem cells are in the presence of pro-inflammatory cytokines (i.e., TNF α , IL-1, IFN γ) they can produce chemokines that result in T-cell migration into these areas [94]. Once T-cells are in close proximity, their activity is suppressed by the production of nitric oxide, IL-10, and TGF- β by these stem cells [92,94]. There is also data to suggest that mesenchymal stem cells can regulate APC activation of T-cells through cell to cell interactions [91]. Although these findings are very exciting, the use of these therapies in DMD patients will require extensive research to demonstrate their clinical efficacy.

Several anti-cytokine drugs, which are already available for use in human patients, are capable of decreasing dystrophic muscle pathology [25,29]. Infliximab (Remicade) and etanercept (Enbrel) have been used to block TNF α in rheumatoid arthritis and Crohn's disease [95]. These drugs have also been shown to greatly improve muscle pathology in *mdx* mice [25,29]. Because TNF α is known to inhibit contractile function of skeletal muscle and induce muscle wasting, blockade of this cytokine is a logical therapeutic option for DMD patients [74]. Interleukin 1 receptor antagonist (Anakinra) could potentially be used in DMD patients and has already been used in the treatment of rheumatoid arthritis and other inflammatory diseases [96]. There are also newly developed drugs that block MCP-1 (Bindarit) and TGF- β (TGF- β antagonist), but they are only in the initial stages of assessment [97,98]. A thorough investigation of the cytokines responsible for regulating the dystrophic disease process and their regulation of the disease time course has not been conducted, but will undoubtedly result in the identification of other targets for the amelioration of DMD. Several anti-cytokine therapies have been shown to improve *mdx* muscle pathology and may be useful treatments for DMD patients.

As described above, the transcription factor NF- κ B may be responsible for muscle wasting [9,47,51,99]. NF- κ B regulates the expression of numerous cytokines which are shown to be

elevated in dystrophic muscle [9,47,51,52], and therefore represents a suitable target for therapy. Epigallocatechin gallate (EGCG), is a component of green tea extract that is a potent antioxidant that can regulate NF- κ B activation [100-102]. EGCG inhibits NF- κ B activation by blocking IKK activity; however, the mechanism through which EGCG regulates this pathway is not fully understood [101,102]. Since NF- κ B activation is sensitive to free radicals, EGCG's ability to reduce their formation may be a key mechanism. Although there are no reports of DMD patients being treated with green tea extract, treated *mdx* mice have increased antioxidant capacity, improved contractile properties, and decreased muscle pathology [103-106]. Another inhibitor of NF- κ B activation is curcumin, a dietary component of the spice turmeric [107]. *Mdx* mice treated with injections of curcumin have reduced NF- κ B activation and iNOS expression. TNF α and IL-1 β levels in the serum of these mice are also reduced [107,108].

The antioxidants, pyrrolidine dithiocarbamate (PDTC) and *N*-acetylcysteine (NAC), have been used to treat human diseases and are reported to block NF- κ B activation [109]. In *mdx* mice PDTC treatment greatly improves force deficit, forelimb strength, and overall body strength [99,110]. These mice have decreased NF- κ B activation and TNF α production in their muscle tissue [99]. Histologically these muscles demonstrate significant decreases in necrosis and increases in regenerating fibers [99]. Similar results are seen in mice treated with NAC [111]. Encouraging results from *mdx* mice indicate that these inhibitors of NF- κ B may provide beneficial treatments for DMD patients and could be put to use relatively quickly.

The development of new therapeutic options would greatly benefit from thorough time course studies in DMD patients and animal models that detail the signaling processes that regulate inflammation in dystrophic muscle. Once key signaling processes are identified, treatments can then be tailored to alter these processes in DMD patients. Nevertheless, several anti-inflammatory therapies could have clinical relevance in the very near future. These include the use of nutritional therapies (i.e., EGCG and curcumin) and anti-cytokine therapies (i.e., infliximab or etanercept). Stem cell therapies hold great promise to both regenerate muscle tissue and suppress inflammation. Once fully developed, this therapeutic approach could ultimately lead to a cure for DMD patients.

Conclusion

Mechanical injury and membrane defects are important pathological factors that impact dystrophic muscle wasting; however, these features do not fully explain dystrophic muscle lesion formation and these changes are reported only after overt disease onset. In contrast, altered cell signaling, elevated immune cell infiltration and expression of immuno-regulatory proteins have been reported in dystrophic muscle before and during the overt onset. The absence of dystrophin and DGC proteins is thought to contribute to altered mechanical integrity of muscle fibers, but has more recently been linked to aberrant cellular signaling. Increased activation of transcription factors (e.g., NF- κ B), in connection with Ca²⁺ influx and ROS production, involved in the expression of inflammatory genes are reported in dystrophic muscle before disease onset. NF- κ B regulates the expression of numerous inflammatory genes and has been indicated as a key factor in the initiation and progression of dystrophic muscle wasting. Increased expression of cytokines and chemokines, which regulate infiltration and activation of immune cell populations are observed in connection to NF- κ B activation. The role of cytokines and chemokines in the pathogenesis of DMD has not been comprehensively examined, but may provide valuable insight into disease mechanisms that regulate muscle pathology. In dystrophic muscle, immune cells contribute substantially to muscle wasting and are known to modulate apoptosis, necrosis, and fibrosis. Early therapeutic interventions, which disrupt immune processes in *mdx* mice greatly decrease muscle pathology and may provide the most beneficial treatments for DMD. Many of these treatments are presently in use to treat other human diseases (e.g., rheumatoid arthritis and Crohn's disease), and therefore could be

applied to DMD relatively quickly when compared to treatments that still require extensive testing (e.g., gene therapy). Although many immune-mediated disease processes have been identified in dystrophin-deficient muscle, characterization of a detailed time course of these mechanisms has been largely neglected in both human patients and in dystrophic models. Specifically, time course studies that profile the inflammatory/immune response in detail during the period before and after initial disease onset will be invaluable to identify new therapeutic targets.

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Figure 1.

The dystrophin glycoprotein complex (DGC) in normal myofibers. Illustration of the link formed between laminin in the extracellular matrix (ECM) and the cytoskeleton by the DGC. Also shown, interaction of DGC components (dystrophin, dystroglycans, dystrobrevin, laminin, sarcoglycans, syntrophin, and sarcospan) with potential signaling proteins (Grb2, SOS, RAS, NOS, CaM, CaMKII), and with stretch activated channels (SAC) through which calcium (Ca) can enter the cell. Reprinted with permission. Evans NP, Misyak SA, Robertson JL, Bassaganya-Riera J, Grange RW. Dysregulated intracellular signaling and inflammatory gene expression during initial disease onset in Duchenne muscular dystrophy. *Am J Phys Med Rehabil* 2009;88:502-522.

Figure 2.

Possible immune cell interactions in dystrophic muscle. Macrophages, CD4⁺, and CD8⁺ cells appear to primarily interact within the muscle tissue of *mdx* mice, whereas in a typical immune response, APC's would engulf antigens and carry them back to draining lymph nodes to interact with CD4⁺ and CD8⁺ T cells. Dystrophic muscle fibers likely produce antigens which are engulfed by macrophages and are activated by additional inflammatory stimuli. In DMD patients, T cells have specific TCR rearrangements that may allow them to interact with APC which express MHC I and II. Therefore, one possibility is that macrophages (which have both MHC I and II proteins on their surface) could induce muscle fiber death through nitric oxide mediated cell lysis or present antigen, bound to MHC II, to CD4⁺ T cells through their T cell receptors. Activated CD4⁺ T cells would then produce cytokines to activate CD8⁺ T cells. When receptors on CD8⁺ T cells come in contact with antigen, they bind to MHC I on the surface of muscle fibers and could induce muscle fiber death.

Figure 3.

Possible pathways resulting in NF-κB activation. (A) In WT muscle fibers the DGC may play key role in regulating or suppressing NF-κB activation but the mechanism for how this happens has not been defined. (B) In *mdx* muscle fibers, the loss of the DGC has been reported to contribute to changes in signaling pathways that increase NF-κB activation. Reprinted with permission. Evans NP, Misyak SA, Robertson JL, Bassaganya-Riera J, Grange RW. Dysregulated intracellular signaling and inflammatory gene expression during initial disease onset in Duchenne muscular dystrophy. *Am J Phys Med Rehabil* 2009;88:502-522.

Figure 4.

Possible interactions of extracellular immune signaling pathways in dystrophic muscle. In DMD and *mdx* (M) muscle tissue, elevated levels of (C) chemokines and (P) pro-inflammatory cytokines are produced, which when released into the ECM can act as chemoattractants for a variety of (I) immune cells. Although the process has not been described in dystrophic muscle, activated immune cells may travel to sites of muscle damage where they can release pro-inflammatory and (A) anti-inflammatory cytokines. The balance between pro-inflammatory and anti-inflammatory cytokines may regulate the level of immune cell activation in dystrophic muscle and resulting muscle fiber death.

Table 1

Infiltrating Immune cells	Peak Age	Peak Infiltration	References
Macrophages	4 - 8 weeks	>80,000 cells/mm ³	[13,14]
T-cells	4 - 8 weeks	>1200 cells/mm ³	[13,42]
Neutrophils	ND	ND	[25]
Eosinophils	4 weeks	>4000 cells/mm ³	[34]
Mast Cells	4 weeks	30 fold increase	[36]

ND, No Data