Published in final edited form as:

Sci Transl Med. 2015 August 05; 7(299): 299rv4. doi:10.1126/scitranslmed.aaa7322.

Immune-mediated pathology in Duchenne muscular dystrophy

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Abstract

Immunological and inflammatory processes downstream of dystrophin deficiency as well as metabolic abnormalities, defective autophagy, and loss of regenerative capacity all contribute to muscle pathology in Duchenne muscular dystrophy (DMD). These downstream cascades offer potential avenues for pharmacological intervention. Modulating the inflammatory response and inducing immunological tolerance to de novo dystrophin expression will be critical to the success of dystrophin-replacement therapies. This Review focuses on the role of the inflammatory response in DMD pathogenesis and opportunities for clinical intervention.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is a genetic disorder of muscle caused by mutations in the *DMD* gene encoding the dystrophin protein on the X chromosome. Dystrophin is a large (427 kD) membrane cytoskeletal protein that imparts structural stability to the plasma membranes of myofibers, so that they are better able to withstand the contraction/relaxation cycles and force generation required of muscle tissue. DMD patients are unable to produce dystrophin. This lack of dystrophin in myofibers leads to contraction-induced membrane damage with release of cytoplasmic contents and stimulation of innate immunity, cycles of myofiber degeneration/regeneration, age-related replacement of muscle by fibrofatty connective tissue, muscle weakness, and, ultimately, death. DMD is among the most common of neuromuscular disorders, due in large part to the high mutation rate of the very large gene (2.3 million base pairs). It is also one of the more rapidly progressive of the neuromuscular disorders: A seemingly healthy young child first shows difficulties keeping up with peers in early school age, then experiences progressive weakness followed by loss of

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ambulation in the second decade, and typically succumbs to the disease due to cardiorespiratory complications within his or her mid-to-late 20s.

Spontaneously occurring mouse (mdx), dog (CXMD), and cat models of DMD have been identified and characterized. These animal models show remarkable variation in the age of onset and severity of the muscle disease. Within an individual animal, specific muscles are differentially affected. Indeed, a notable feature of both DMD and its animal model counterparts is the variable response of certain muscles to the same biochemical defect, with some showing a hypertrophic rather than a wasting phenotype (1). The species- and muscle-specific involvement is thought to be driven by differences in the response to muscle damage and repair, with inflammation playing a major role.

The extent of muscle pathology generally correlates with decreased muscle function. DMD fetal muscle shows little evidence of pathology, despite the marked dystrophin deficiency at the myofiber plasma membrane. However, soon after birth, there is strong activation of multiple components of the innate immune system before the onset of clinical symptoms, including altered signaling via Toll-like receptors (TLR4, TLR7) and via nuclear factor κB (NF-κB), and expression of major histocompatibility complex (MHC) class I molecules on muscle cells (which do not normally express MHC class I). There is increasing evidence that membrane instability and associated release of cytoplasmic contents into the extracellular space mediate this chronic activation of the innate immune system and associated inflammatory response. A second pathological process, which is superimposed on the chronic proinflammatory state, is that of segmental degeneration and regeneration of myofibers. In this process, fibers (singly or in groups) are infiltrated by neutrophils and phagocytosed by macrophages. Meanwhile, resident myogenic stem cells are activated and differentiate into myoblasts, and regeneration of the myofiber occurs within the preexisting basal lamina. As the regenerated myofibers remain dystrophin-deficient, this leads to successive focal bouts of degeneration and regeneration, with a specific temporally staged pattern of inflammatory infiltrates. Although such bouts of degeneration and regeneration are successful in the healing of wild-type muscle, they fail to heal DMD muscle. Ultimately, with increasing age, the interplay between chronic activation of innate immunity and asynchronous and neighboring bouts of degeneration and regeneration combine to yield a poorly orchestrated repair response that may itself drive disease progression.

DYSTROPHIN-DEFICIENT SKELETAL MUSCLE: LOSS OF IMMUNOLOGICAL PRIVILEGE

Skeletal muscle tissue has unique features that appear to result in a relatively low capacity to generate localized immune responses. The tissue has a low number of resident dendritic cells, mast cells, and other proinflammatory cells per gram of tissue. It is a preferred site of immunization because of such immunological privilege, which confers a very low rate of abscess and granuloma formation compared to the subcutaneous route of administration. Underlying such observations, muscle as a site of immunization has also been found to be less sensitive to adjuvants, with less necrosis and irritation compared to subcutaneous delivery (2).

Critical aspects of the normal biology of muscle necessitate its immune privileged status, a phenomenon that is highlighted by its failure in DMD. As part of normal intensive muscle activity, large syncytial myofibers show leakage of cytoplasmic contents into the extracellular milieu, with muscle cytoplasmic enzymes (creatine kinase) appearing in blood and microscopic and cellular evidence of the unrestricted flow of cytoplasmic content across membranes. It is well established that leakage of cell cytoplasm into the extracellular milieu is a potent trigger of innate immune responses, including the binding of damage-associated molecular pattern (DAMP) molecules (for example, heat shock proteins and nucleic acids) to TLRs with subsequent inflammasome formation (3). Although leakage of cytoplasmic contents in actively exercising normal muscle (particularly eccentric or lengthening contractions) activates TLRs, the relative immune privilege coupled with rapid membrane repair and intact dystrophin appear to limit and quickly resolve inflammation. Moreover, given that there is no constitutive MHC class I expression on skeletal muscle cells, there is no progression from innate to adaptive immune responses with exercise-induced injury.

Upon trauma or other acute damage, muscle can become necrotic. However, muscle is also a regenerative tissue with myofiber necrosis resulting in a highly orchestrated sequence of cellular events involving different subsets of inflammatory infiltrates that unfolds over about 2 weeks. First, neutrophils infiltrate the necrotic area within hours, followed by M1 (inflammatory) macrophages within days. Subsequently, M2 (modulatory) macrophages gradually replace the M1 macrophages. As with subnecrotic damage, the interactions of myofibers with the immune system are highly staged and resolve quickly with return of function in normal muscle. The nature of muscular activity is captured in the term “bout effect,” conveying the concept that all types of damage to normal muscle are typically episodic and have a well-orchestrated resolution of inflammation and repair (4).

In DMD, on the other hand, dystrophin deficiency leads to chronic membrane instability with a continuous release of TLR ligands into the extracellular milieu (Fig. 1). Persistent membrane instability leads to self-sustaining activation of the innate immune response. Proinflammatory cytokines induce constitutive MHC class I and II expression on muscle cells, recruitment of T and B cells, and generation of an adaptive immune response in the muscle milieu. This proinflammatory micro-environment is often superimposed on the neutrophil and macrophage infiltrations induced by successive courses of myofiber degeneration and regeneration. In contrast to the full resolution of a single bout of inflammation and repair of normal muscle, dystrophin-deficient muscle loses the bout effect. Neighboring fibers or groups of fibers enter the necrotic stage at different times in the 2-week degeneration/regeneration cycle (asynchronous regeneration), thus sustaining a chronic inflammatory state, which, in turn, creates a more proinflammatory environment with activation of innate immune pathways, and evidence of increased antigen presentation (5) (Fig. 2).

As noted above, specific muscles show marked variability in pathology, both within an individual biopsy, between muscles in a human patient, and among species. One potential explanation for this variability is the extent to which inflammation is provoked. The interplay between the effects of chronic stimulation of innate immunity by membrane leakage (primary defect) and bouts of asynchronous degeneration/regeneration may be kept

at a low level in some muscles, but in others may synergize to become destructive. Many of these immunopathological features of dystrophin-deficient muscle vary depending on muscle group and age (6–10). For example, in both dystrophin-deficient humans and dogs, the sartorius muscle is spared; although the basis for such sparing is uncertain, studies suggest that multiple interacting pathways may have protective roles (7). Notably, there are additional aspects of pathophysiology, over and above inflammation, that likely contribute to variability including induction of compensatory pathways (utrophin, myostatin, and others), type of activity of specific muscle groups (for example, stress on myofibers), and others.

WHERE IT BEGINS: ACTIVATION OF THE INNATE IMMUNE RESPONSE

Pattern recognition receptors such as TLRs detect not only materials originating from microbes but also those arising from damaged cells of the host. Self-molecules released from damaged cells that activate TLRs are those displaying DAMPs. DAMPs arising from damaged or necrotic muscle include heat shock proteins, high-mobility group box 1 (HMGB1) proteins, and reactive oxygen species as well as nucleic acids.

In dystrophin-deficient muscle, myofiber-derived RNA molecules may be the most potent DAMPs as TLR7 (specific for single-stranded RNA) expression is up-regulated at very early stages of the disease in muscle, in infiltrating mononuclear cells, and in blood vessels of presymptomatic DMD patients (<1 year of age) (Fig. 1) (11). TLR7 up-regulation is accompanied by activation of inflammatory signaling pathways, leading to elevated expression of MHC class I and II molecules and complement factors D and H, as detected by mRNA profiling and immunohistochemistry studies. Expression of subsets of this early inflammatory gene cluster (HLA-DQβ, HLA-DRβ, and complement factor D) increases with age in presymptomatic DMD patients, but most remain stably up-regulated throughout the disease course (11). Expression of other TLRs should be investigated in DMD given that TLR1 to TLR4 and TLR7 to TLR9 were found to be up-regulated in the mdx murine model of DMD (12). Also consistent with a key role for TLR7-induced inflammation in DMD muscle is the highly up-regulated NF-κB signaling pathway that acts downstream of TLR7 (Fig. 1) (11). NF-κB activation is known to induce expression of downstream mediators of inflammation including cytokines, chemokines, and adhesion molecules, which recruit innate immune cells to infiltrate the tissue and activate local cells at the damaged tissue site.

COMPLEMENT ACTIVATION: A PLAYER IN DYSTROPHIN-DEFICIENT MUSCLE?

The complement system, part of the innate immune system, links the capacity of antibodies and phagocytic cells to clear opsonized cells and proteins, promotes chemotaxis, and targets cells for lysis by the membrane attack complex. Three biochemical pathways lead to complement activation: the classical, alternative, and lectin pathways (13). The classical and alternative pathways are triggered by the C1 complex and hydrolysis of C3, respectively, whereas opsonins, mannose-binding lectins, and ficolins activate the lectin pathway. Complement-mediated damage may be an underappreciated element of muscle pathology and myofiber damage in DMD. Indeed, in DMD and other inflammatory myopathies, complement membrane attack complexes with complement components C5b-C9 have been

detected (using a monoclonal antibody specific for the neoantigens of the attack complex) on necrotic muscle fibers, but not on nonnecrotic fibers (14). Immunoglobulin was not detected on such fibers, indicating that complement was activated by the alternative pathway, a finding further supported by the up-regulation in DMD of complement factors B and D and properdin, which are known to interact with C3. Moreover, the C5a cleavage fragment generated during activation of the alternative pathway is chemotactic for macrophages and neutrophils, the former being highly abundant in DMD muscle. Thus, agents that inhibit formation of the complement membrane attack complex have the potential to diminish muscle necrosis in DMD. These include eculizumab, a monoclonal antibody that specifically binds to C5, preventing generation of the terminal attack complex. Eculizumab is approved for use in paroxysmal nocturnal hemoglobinuria (15). The role of C3, however, is not clear. Although C3 was localized to all necrotic fibers in DMD, usually well above background levels, the receptor for C3 has been found to negatively regulate TLR7-mediated signaling (by degrading MyD88). Thus, C3 may be serving an anti-inflammatory role in this setting (16). Therefore, it is puzzling that ablation of C3 in mdx mice failed to have an effect on muscle pathology. This finding may reflect the overall lesser degree of muscle pathology in mdx mice than in DMD patients or could indicate species-specific differences in activity of this complement element (17).

CYTOKINES AND CHEMOKINES

Cytokines and chemokines often serve a modulatory role at the interface of innate immune activation and adaptive immunity (Fig. 1). A number of chemokines have been associated with DMD pathology, as determined by studies of gene expression and immunohistochemistry. Studies profiling gene expression in DMD muscle indicate that four chemokines—CCL14, CCL2, CXCL12, and CXCL14—are up-regulated (18). In immunohistochemistry studies, CXCL12, CXCL11, and CCL2 all showed increased expression in the blood vessel endothelium of DMD patients. CXCL14 was not evaluated in DMD by this method (19). CXCL14 has been shown to be a chemoattractant for and an activator of dendritic cells, although its receptor is currently unknown (20). Activated dendritic cells, marked by positive staining for or up-regulation of CD86, DC-LAMP, and HLA-DR, are prominent in DMD muscle and may well reflect upstream activation of CXCL14, but further studies are needed to better define this mode of dendritic cell activation.

Intriguingly, mdx mouse muscle shows expression of an array of chemokines that are quite distinct from those in DMD, which may help to explain the differences in pathogenesis between the two diseases (21). Especially intriguing is the finding that resident regulatory T cells (T_{regs}) in muscle express CCR1, a receptor for CCL5 and CCL7 that is expressed in mdx muscle but not in DMD muscle (22). Thus, the milder disease phenotype in mdx mice could be attributable to better recruitment of cells with immunosuppressant properties.

CXCL12 is present on normal muscle fibers and is highly expressed in regenerating muscle fibers in DMD. CXCL12, acting through CXCR4, attracts the majority of leukocytes and lymphocytes including macrophages, $CD4^+$ T cells, and $CD8^+$ T cells, the very portrait of the cellular infiltrate in DMD muscle (19). However, the CXCL12/CXCR4 axis is also

important in muscle regeneration as demonstrated in mice, where it regulates migration of both proliferating and terminally differentiated muscle cells and is necessary for proper fusion of muscle cells (23). In this regard, plerixafor, the first CXCR4 antagonist approved by the U.S. Food and Drug Administration for mobilization of hematopoietic stem cells in the setting of malignancy, is a potential investigative treatment option to decrease inflammation in DMD (Table 1). However, the reduction in inflammation may come at the cost of inhibiting the mobilization of satellite cells important in muscle repair (24).

Tumor necrosis factor–α (TNF-α) is associated with inflammation in numerous autoimmune and inflammatory settings, but whether it contributes to inflammation in DMD is not clear. Although it has been detected in 62% of DMD patient biopsies (19), it has not been cited in the list of top inflammatory mediators in more recent gene expression profiling studies (18). Moreover, TNF-α appears to have a dichotomous role in muscle physiology as illustrated by studies in mdx mice: mice lacking both dystrophin and TNF-α showed decreased muscle mass and evidence of accelerated pathological progression (in diaphragm muscles). This is consistent with the finding that TNF-α has a prominent role in promoting muscle maintenance and repair. In contrast, mdx mice treated with infliximab (that express TNF-α during early development and as neonates) had a delayed appearance of muscle pathology, potentially supporting the use of TNF-α blockade to slow disease progression (25) (Table 1). Additional studies are therefore needed to clarify the time course and correlation of TNF-α expression with pathological changes in DMD to better understand whether blockade with approved TNF-α inhibitors should be considered further.

Other inflammatory cytokines including interleukin-1β (IL-1β), interferon-γ (IFN-γ), IL-6, and type I IFNs do not appear prominent in gene expression arrays, but several proteins induced by IFN-γ are indeed up-regulated. Moreover, although direct evidence that IFN-γ contributes to inflammation in DMD is lacking, genetic deletion of IFN-γ in mdx mice attenuated myofiber injury and increased running time on a treadmill (26). In addition, specific deletion of T_{regs} led to enhanced expression of an IFN-γ genetic signature, suggesting that IFN-γ may indeed have a role in the immunopathology of DMD and thus warrants further study. Although detection of IL-1β and IL-6 in tissues by immunohistochemistry and reverse transcription polymerase chain reaction (RT-PCR) is variable, dystrophin-deficient primary muscle cells have been shown to produce substantial amounts of IL-1β (27). Importantly and unexpectedly, in mdx mouse studies in which IL-6 was blocked with a monoclonal antibody, muscle pathology worsened, suggesting a paradoxical anti-inflammatory role for IL-6 at least in the mdx mouse (28) (Table 1). Insight into why IL-6 may diminish inflammation rather than promote it comes from studies demonstrating that signaling through the IL-6R induces expression of IL-4R on macrophages, which is essential for them to switch from a proinflammatory M1 type to an M2 type that boosts tissue repair (29).

INNATE IMMUNITY AND THE ROLE OF MACROPHAGES

An increasing appreciation of the diverse nature of infiltrating macrophages in mdx mice has led to consideration of additional strategies to minimize damage and promote healing of muscle in DMD (Fig. 1). M1 macrophages (classically activated, proinflammatory) play a

strong role in muscle injury in mdx mice early in disease because of the nitric oxide–mediated cytolytic capacity of such cells. Elimination of macrophages early in mdx disease produced a reduction in muscle lesions (30). Later in the course of disease, IL-10–induced M2c macrophages (activated by an alternative pathway) deactivate these proinflammatory M1 cells, resulting in tissue repair (31). Thus, suppression of a proinflammatory response in mdx mice by IL-10–producing cells including T_{regs} may be critical for permitting the generation of reparative M2 macrophages (26). Treatment with IL-10 would therefore appear worthy of investigation, although to date there is no evidence of its efficacy in studies of autoimmunity (32). However, this may be due to failure to deliver the agent in sufficient amounts to the afflicted cells or tissues when administered systemically. Whether a sufficient dose of IL-10 would enable its access to muscle if given systemically is not known but could be tested in animal models. Direct injection or gene-induced expression in muscle is also a possibility, at least to evaluate proof of concept.

CHRONIC TRANSFORMING GROWTH FACTOR–β SIGNALING AND FIBROSIS IN DMD MUSCLE

As with many chronic inflammatory conditions, DMD muscle shows age-related increases in transforming growth factor–β (TGF-β) and associated fibrotic replacement of the tissue. The regulation of the immune system by TGF-β is highly complex and context-dependent (33), making development of TGF-β inhibitors difficult. However, TGF-β is the one factor that all studies agree is consistently up-regulated in DMD, with expression of TGF-β and TGF-β receptors (TGFBR) associated with symptomatic disease (34). Patients with DMD show higher expression of TGF-β1, with peak expression between 2 and 6 years of age, and more fibrosis than do patients with Becker's muscular dystrophy or other muscular dystrophies (35).

When bound by TGF-β, its receptor canonically signals through Smad2/3 (TGF-β Smads) and Smad4 (36). However, in the context of TGF-β overexpression in disease settings, Smad2/3 aberrantly associates with Smad1 and Smad5 (bone morphogenetic protein Smads) forming "mixed Smad" complexes (37) that have been observed in mouse models of pathological fibrosis. Indeed, Smad1 and Smad5 are up-regulated and activated in DMD (38).

TGF-β is secreted as an inactive protein that requires additional processing to assume its active form. It is worth noting that matrix metalloprotease 2 (MMP2), known to activate TGF-β (39), is up-regulated in DMD muscle, suggesting a possible role for MMP2 in activating TGF-β in this setting (40). TGF-β may also be activated in response to pH changes, reactive oxygen species, and matrix rigidity (41). The part played by these factors in converting TGF-β to its active form in DMD is not known. Also supportive of a key role for TGF-β1 pathways in DMD disease progression is the identification of two genetic modifier loci (*SPP1* and *LTBP4*) both known to alter TGF-β1–mediated pathways and DMD disease severity (42, 43). Indeed, data about genetic modifiers suggest that TGF-β1 pathways may be more important than any other downstream pathway in driving the progressive muscle wasting and weakness observed in DMD.

Recent data on regenerating microenvironments in wild-type mouse muscle have shown that connective tissue remodeling is a normal part of a “bout” of muscle regeneration (5). However, with asynchronous bouts of regeneration in neighboring microenvironments, TGF-β1 becomes constitutively activated, leading to continuous connective tissue remodeling eventually resulting in fibrosis (Fig. 2) (5). Thus, mouse model data also support the importance of TGF-β1 pathway modulation in the chronically inflamed muscle of DMD patients. A key feature of this model is that the normally tightly orchestrated influx of neutrophils, M1 macrophages, and then M2 macrophages during normal myofiber regeneration is perturbed because of inappropriate crosstalk between neighboring asynchronously remodeling myofibers in DMD muscle (Fig. 2).

Myostatin or GDF8, a member of the TGF-β superfamily, negatively regulates muscle differentiation and growth. Spontaneous mutations in humans or deliberate knockout of this factor in mice induces muscle hypertrophy; hence, this factor has been targeted as a way to improve muscle function (44). In the mdx mouse and in DMD skeletal muscle, myostatin mRNA expression appears to be down-regulated (45). This may represent an adaptive response to maintain muscle mass and to rescue dystrophic muscle. Further investigations into myostatin protein expression and its relationship to the inflammatory response, fibrosis, and loss of muscle mass in DMD are needed to evaluate whether this protein could prove to be a therapeutic target.

OTHER FACTORS CONTRIBUTING TO FIBROSIS

The increased expression in DMD muscle of collagen species, connective tissue growth factor, osteopontin (46), and the tissue inhibitor of metalloproteinases-1 (TIMP1), which has been linked to development of pulmonary and liver fibrosis (46, 47), may similarly contribute to fibrosis in DMD muscle. Osteopontin is highly expressed in muscle and by infiltrating T cells in the mdx mouse, and its mRNA is up-regulated in DMD (48). Knocking out osteopontin in the mdx mouse reduced fibrosis and enhanced muscle strength through a combined effect that resulted in reduced TGF-β and increased T_{reg} infiltration. However, as with other proinflammatory agents, osteopontin may have a positive effect on muscle regeneration as shown in vitro (49). Agents that antagonize or neutralize osteopontin will need to be evaluated in animal models of DMD (Table 1).

“ORPHAN” MARKERS

In a study of DMD muscle biopsies (50) using an Affymetrix microarray (HuGeneFL) and confirmed by immunohistochemistry, factor XIIIa was shown to be overexpressed concomitantly with HLA-DRα by activated dendritic cells. The function of factor XIIIa, a tissue transglutaminase, in activated dendritic cells is not clear, but it may contribute to collagen formation and fibrosis in DMD muscle. More data are clearly needed to identify the role, if any, of this factor in disease pathology (51). Although muscle cells do not express MHC class II antigens under normal physiological conditions, they may do so in some inflammatory myopathies and thus are important markers of inflammation (52, 53). Moreover, the dendritic cells and macrophages invading inflamed muscle do express MHC class II, and both of these populations can therefore serve as antigen-presenting cells for

$CD4^+$ MHC class II–restricted T cells that provide help to $CD8^+$ cytolytic cells that are specific for muscle-derived peptides (for example, dystrophin) in the context of MHC class I.

Thrombospondin 4 (TSP-4) is up-regulated in DMD by as much as 15-fold. A role for TSP-4 in inflammation has been shown in mouse models of atherosclerosis in which TSP-4 was ablated. These studies revealed that TSP-4 activates endothelial cells and macrophages and promotes macrophage adhesion and migration (54). TSP-4 may well contribute to inflammation in the setting of DMD, but this requires further investigation.

MUSCLE MAST CELLS: EXACERBATION OF CHRONIC INFLAMMATION

Mast cells are typically associated with allergic inflammation, particularly in lung and skin. In normal muscle, the few resident mast cells are localized around arteries and veins, and are small and quiescent. Dystrophin-deficient muscle exhibits marked proliferation of mast cells throughout the endomysial connective tissue that surrounds myofibers; these mast cells are typically large or degranulated, and are chronically activated (55). This suggests that the chronic inflammatory state in mdx mouse muscle and DMD muscle leads to recruitment of mast cells from the circulation or induces proliferation of tissue-resident populations, which then degranulate in the proinflammatory milieu. Consistent with this model, mild damage to normal skeletal muscle induces mast cell recruitment from the peripheral circulation (56).

The proteases and cytokines released by degranulation of mast cells localized in endomysial connective tissue might be expected to exacerbate the already fragile dystrophin-deficient plasma membrane, and this is indeed the case (57). Also consistent with a role for mast cells in exacerbating both the proinflammatory state and membrane damage is the finding that engineering mdx mice to produce more mast cells led to a worsening of disease pathology including increased fibrosis (58). In contrast, pharmacological inhibition of mast cell production in mdx mice ameliorated the disease phenotype (Table 1) (59, 60). Thus, a potential therapeutic strategy may be to counter mast cell degranulation.

ADAPTIVE IMMUNITY: T CELL INVOLVEMENT IN DMD

The roles of $CD4^+$ T cells and $CD8^+$ T cells in DMD muscle pathology have been examined critically in humans and in animal models. Steinman and colleagues in 1994 discovered that the T cells in the muscle of 12 DMD patients expressed the Vβ2 T cell receptor (TCR) and also predominantly the Jβ1.3 segment (61). Additionally, in 5 of 12 patient samples (all HLA-A A2- or A1-positive), there was a common amino acid motif, RVSG, in the CDR3 region of Vβ2. This proclivity for Vβ2 TCR sequences was not observed in muscle from patients with other muscle diseases such as polymyositis (which had a predominance of Vβ15 TCR) (61). Although the antigenic specificity of these T cells was not evaluated, these studies suggest that among $CD8^+$ T cells (assumed to be such by HLA-A restriction) infiltrating DMD muscle, there may be selection for a subset of T cells that are specific to a restricted set of muscle antigens. Oligoclonality of T cell subsets was also observed in the mdx mouse model, with Vβ8.1/8.2 being the predominant population in muscle but not in spleen (48), further supporting antigen restriction of the T cell response in dystrophin-

deficient muscle. It is likely that these oligoclonally derived T cells are specific for dystrophin peptides in the context of MHC class I and II expressed on revertant myofibers. Nonetheless, specificities for other muscle proteins should be investigated because it is possible that prolonged inflammation has unleashed an autoimmune response that has spread to other epitopes in different muscle proteins.

In other patient studies, dystrophin-specific $CD8^+$ T cells were detected in the peripheral blood lymphocyte population of patients with DMD and were presumed to originate through exposure of T cells to expression of a partial dystrophin molecule on revertant myofibers (62). However, these dystrophin-specific T cells were evaluated neither for their use of restricted Vβ2 CDR3 segments nor for the presence of the RVSG CDR3 motif.

In mdx mice, elimination of either $CD4^+$ or $CD8^+$ T cell populations had a salutary effect on muscle histopathology: there was a 61% reduction in histopathology after $CD4^+$ T cell depletion and a 75% reduction after $CD8^+$ T cell depletion (63). Both perforin-mediated and non–perforin-mediated mechanisms appear to be involved in the damage caused by $CD8^+$ T cells. One mechanism by which $CD8^+$ T cells potentially contribute to muscle damage involves recruitment of inflammatory cells such as eosinophils, an immune cell type that was reduced in mdx muscle after $CD8^+$ T cell depletion in mice (64). Critically, T cell depletion [by breeding mdx mice onto a SCID (severe combined immunodeficient) background] significantly reduced both TGF-β expression as well as fibrosis in the muscles of these animals (65). The prominence of $CD8^+$ T cell–mediated pathology in DMD is further supported by the effect of steroid treatment in DMD patients. Improvements in muscle strength in DMD patients treated with low-dose (0.75 mg/kg per day) or high-dose (1.5 mg/kg per day) steroids correlated with a reduction in total T cell number, particularly in $CD8^+$ T cells, as well as a reduction in the number of muscle fibers focally invaded by lymphocytes (66).

REGULATORY T CELLS IN MDX MOUSE MUSCLE AND DMD MUSCLE

Many of the $CD4^+$ T cells infiltrating DMD muscle appear to be regulatory in nature, that is, T_{regs}. In the mdx mouse model, the number of T_{regs} increases in dystrophic muscle at early and late time points in disease, and, similar to DMD muscle, shows evidence of clonal expansion suggesting antigen specificity. Eliminating the T_{reg} population with the highest expression of CD25 by treating mdx mice with an anti-CD25 monoclonal antibody worsened disease by histopathological assessment, induced a modest increase in creatine kinase (a muscle-specific enzyme released after muscle damage), and up-regulated genes encoding osteopontin and connective tissue growth factor, both of which are associated with fibrosis (22). In contrast, administration of antibody-complexed IL-2 (IL-2C) expanded the number of T_{regs}, reduced inflammation and muscle damage, and caused a marked reduction in creatine kinase (67) (Table 1). The activity of T_{regs} in these studies was attributed to T_{reg}-mediated secretion of IL-10, an immunosuppressive cytokine. These findings indicate that T_{regs} likely dampen both the inflammatory and fibrotic processes affecting DMD muscle. T_{regs} may also have direct effects on muscle growth and regeneration through secretion of amphiregulin, an epidermal growth factor family member whose receptors are expressed on muscle satellite cells that are critical for muscle regeneration (22).

Increased numbers of T_{regs} in DMD muscle have been reported and correlate with an increase in IL-10 expression as measured by qPCR (quantitative real-time fluorescence PCR) (67). Thus, indiscriminate elimination of $CD4^+$ T cells in DMD may prove damaging rather than beneficial. Rapamycin spares and even induces production of T_{regs} in diverse clinical settings (68, 69). This drug also preserves T_{regs} in mdx mouse muscle while reducing $CD4^+$ and $CD8^+$ effector T cells and decreasing muscle fiber necrosis (70). The Akt/mTOR (mammalian target of rapamycin) pathway targeted by rapamycin is a critical regulator of many muscle phenomena including hypertrophy, atrophy, and fiber size, and its down-regulation has the potential to negatively affect muscle regeneration. Bearing in mind the requirement for Akt/mTOR signaling in muscle regeneration, rapamycin treatment reduced muscle fiber regeneration in mdx mice, but overall had favorable effects on disease due to its immunomodulatory capabilities (70). Rapamycin may also have an overall positive impact on autophagy, which fails in DMD muscle due to chronic activation of the Akt/mTOR pathway (Table 1) (71, 72). Treatment with low-dose IL-2 enhanced T_{reg} expansion in the settings of graft-versus-host disease and vasculitis induced by hepatitis C virus infection (73, 74). Together with treatment of mdx mice with antibody-complexed IL-2, these studies provide a rationale for considering IL-2 as a treatment for DMD.

THERAPEUTIC OPPORTUNITIES IN DMD

Glucocorticoids

Steroid therapy is a mainstay of treatment for DMD patients with beneficial effects on muscle strength, muscle mass, and a delay in disease progression (75). Such effects are assumed to occur through the immunomodulatory activities of these drugs. This has been substantiated in part in a study of DMD patients treated with steroids. Improvements in muscle strength in DMD patients treated with low-dose (0.75 mg/kg per day) or high-dose (1.5 mg/kg per day) steroids correlated with a reduction in total T cell number, particularly $CD8^+$ T cells, as well as in the number of muscle fibers focally invaded by lymphocytes (66). No changes were observed in B cells, $CD4^+$ T cells, macrophages, or muscle fiber necrosis. A recent study of dystrophin-specific T cell immunity in DMD as a function of steroid treatment—DMD patients received either prednisone (24 patients) or deflazacort (29 patients) compared to no treatment for patients (19) or healthy controls (21 individuals)—confirmed the correlation between steroid treatment and a reduction in muscle-specific T cells. There was a substantial reduction in dystrophin-specific T cells [measured by IFN-γ ELISPOT (enzyme-linked immunospot)–positive peripheral blood lymphocytes, which may have underestimated their frequency] in steroid-treated DMD patients: 0% dystrophin-specific T cell responses in healthy controls; 9 of 17 (53%) dystrophin-specific T cell responses in untreated DMD patients; and 11 of 53 (21%) dystrophin-specific T cell responses in steroid-treated DMD patients (5 of 29 with deflazacort; 6 of 24 prednisone) (Table 1). Deflazacort appeared to be more effective than prednisone in this study. Moreover, older subjects (in their teens) had a higher incidence of dystrophin-specific T cells, which also decreased after steroid therapy. There was an 82% response rate in the treatment-naïve group and a 55% response rate in the steroid-treated group by age 20. This higher incidence may be due to the increased frequency in expression of dystrophin by revertant myofibers as DMD patients advance in age (62). Studies of a new steroid-like molecule (VBP15) in mdx

mice suggest that the ability of glucocorticoids to block the master transcription factor NF-κB is crucial for steroid efficacy (transrepression). On the other hand, steroid action on gene promoters (transactivation) may drive the side effects seen in DMD patients and other patient populations treated chronically with steroids (Table 1) (76). VBP15 also shows membrane stabilization properties that may counteract dystrophin deficiency.

Glucocorticoid treatment clearly mitigates symptoms of DMD and has added years of mobility for patients. One might expect that glucocorticoids would show efficacy in a certain subset of the 43 genetic subtypes of muscular dystrophy (www.musclegenetable.fr) given that they all share inflammation as a key symptom (both chronic stimulation of innate immunity, and cycles of degeneration/regeneration). However, there have been very few clinical trials of glucocorticoids in other types of muscular dystrophy, due in large part to the rarity of these conditions and the variability in presentation and disease progression. Anecdotal evidence suggests that some forms of muscular dystrophy (for example, FKRP deficiency) may be responsive to steroids, but that such treatment may be detrimental in others (for example, dysferlin deficiency) (77). There is no convincing explanation for these differences.

Other immune suppressants

Steroids have activities apart from immunosuppression that appear to contribute to their activity in DMD, as other immunosuppressant agents generally have failed to affect muscle function. In a randomized controlled clinical trial, azathioprine reduced the cellular infiltrate in DMD muscle and yet had no effect on measures of muscle function, either alone or in combination with low-dose steroids (78). In a randomized double-blind study of cyclosporine A (CysA), administered either alone or together with steroids, the agent failed to improve muscle strength or boost improvements beyond those of steroid treatment alone, although effects on the cellular infiltrate were not assessed in this study (Table 1) (79). However, in another nonrandomized placebo-controlled study in which the muscle characteristics of DMD patients showed a defined decline over 4 months, CysA treatment halted the decline and patients showed improvements in muscle strength with a larger dose (5 mg/kg versus 3.5 to 4 mg/kg) than that used in the randomized controlled study (80). The primary effect of CysA may be due to diminished muscle cell apoptosis (81), but this is not known in the context of DMD. In canine DMD, treatment with CysA and prednisone demonstrated a mixed outcome with improvement in some muscle characteristics but worsening of others. Improvements were observed in motor scores, gait analysis, and possibly contracture relief, but these were offset by the worsening of force measurements (although this may be due to possible measurement artifact) and worsening of histopathology (with an increase in calcified myofibers) (82).

Of critical importance to such studies is the effect of various immunosuppressive agents on induction, proliferation, and activity of T_{regs}. Most studies of CysA in diverse clinical settings indicate that CysA exerts an adverse impact on T_{regs} and may obliterate the beneficial effects of this crucial T cell population in DMD. In this regard, the immunomodulatory agent rapamycin might be a better therapeutic option for DMD patients. Studies in mdx mice have shown that rapamycin ameliorates the dystrophic phenotype by

suppressing the accumulation of effector T cells in muscle but leaving T_{reg} responses intact (70). Other recent studies using IL-2/anti–IL-2 antibody immune complexes suggest that targeting the IL-2 pathway to increase T_{reg} numbers and function may ameliorate the severity of muscular dystrophy (Table 1) (67).

Targeting innate immunity

To block the contribution of the innate immune response to disease, there are agents that inhibit activation of TLR7 and downstream signaling including the lysosomal inhibitor chloroquine (83) and immunoregulatory oligodeoxynucleotide sequences (84, 85). Blockade of TLR7 and TLR9 signaling using systemically administered oligonucleotide antagonists reduced the inflammatory infiltrate in mdx mouse muscle and damage to muscle as indicated by decreased creatine kinase and improved muscle function (Table 1) (12). In addition, inhibition of the NF-κB pathway ameliorated the dystrophic phenotype and improved muscle function in mdx mice (86–88).

The CXCL12/CXCR4 axis may prove to be a reasonable target for reducing the inflammatory response, but as with other factors that are proinflammatory, caution is needed when considering its blockade. In DMD, it is possible that CXCR4 contributes to trafficking of inflammatory cells from blood to muscle, but therapeutic targeting of CXCR4 with agents such as plerixafor, an approved CXCR4-specific antagonist (Table 1), may also interfere with muscle regeneration (89). Blockade of IL-6 is not warranted in DMD, at least based on data from the mdx mouse model. Although there are approved agents to block IL-1, it is not clear that such treatment would be appropriate without further data substantiating that this cytokine does have an impact on disease progression.

Targeting TGF-β

As with other factors implicated in the pathophysiology of DMD, TGFβs have opposing activities that are specific to disease evolution (90). Although strongly associated with fibrosis, TGF-β also has immunosuppressant activity and is a critical factor in the generation and potentially the maintenance of T_{regs} (91). However, the importance of TGF-β to the muscle-resident T_{regs} present in mdx mouse muscle (22) that suppress inflammation is not known (67). Neutralization of TGF-β early in disease may increase inflammation, preclude generation or expansion of T_{regs}, and increase muscle degeneration, but later in disease, such neutralization may limit fibrosis. A case could be made for treatment with TGF-β blockers early in disease in the context of a combination therapy that decreases cellular activation and inflammation (via NF-κB pathways). This would allow the anti-fibrogenic activity of TGF-β blockade to predominate while fostering conditions for T_{reg} development and expansion. However, a recent study showed that the proliferation of T_{regs} in the presence of TNF-α is potently inhibited by TGF-β (92). Conditional knockout of TGF-β1 in $FoxP3^+$ T_{regs} resulted in increased numbers of these cells in the lymph nodes (93), suggesting that, under some circumstances, TGF-β antagonism might actually expand the T_{reg} population. The impact of TGF-β blockade on the induction and activity of T_{regs} in DMD or its animal models requires more study.

Further support for the reduction of TGF-β as a therapeutic strategy comes from studies in mdx mice and in the spontaneously arising canine model of DMD, golden retriever muscular dystrophy (GRMD). Fibrosis and symptomatology occur early in the dog disease, more closely mimicking human DMD than does the mouse model, and are associated with an increase in TGF-β1 expression (94). In mdx mice, antibody-mediated depletion of TGF-β resulted in a reduction of connective tissue in the diaphragm as well as a large increase in $CD4^+$ T cells (95). It is not clear whether these $CD4^+$ T cells are effector or regulatory in nature given the potential importance of TGF-β in the generation of T_{regs}. In additional mdx mouse studies, treatment with losartan, an angiotensin II type 1 receptor antagonist that attenuates TGF-β–mediated signaling in multiple animal models, diminished fibrosis and improved muscle performance (96). Preliminary studies in DMD patients treated by blocking the angiotensin II type 1 receptor with losartan or the angiotensin-converting enzyme (ACE) with lisinopril showed improved cardiac ejection fractions (97), although the improved contractility may be at least partly attributable to afterload reduction. Moreover, although a control group was lacking, spontaneous improvement in cardiac ejection fraction in this setting is highly unlikely. Only one patient developed signs of potentially heightened immunoreactivity (allergic reaction) potentially due to the effect of diminished TGF-β on T_{reg} development and stability.

The major concern with TGF-β antagonism involves the potential for generation of widespread inflammation, autoimmunity, or cardiovascular complications, as has been observed in TGF-β knockout mice (98, 99). However, in human clinical trials of TGF-β blockade, these outcomes have not been observed (100, 101). Indeed, given the vast experience with ACE and angiotensin II type 1 receptor blockers that antagonize TGF-β, such concerns are at least partially alleviated, although the extent to which TGF-β activity is blocked by these agents at the doses used to treat hypertension is not clear. Thus, approved drugs including angiotensin II type 1 receptor antagonists, such as losartan and candesartan, and ACE inhibitors, such as lisinopril, should be repur-posed and tested for their ability to reduce TGF-β–mediated fibrosis in DMD (Table 1). However, ACE inhibitors may be less desirable because they preclude angiotensin signaling through angiotensin II type 2 receptor, which mediates beneficial activities such as vasodilation, natriuresis, inhibition of renin biosynthesis, and protection of the kidney from inflammation and ischemic injury (102). Preliminary studies of these agents in DMD suggest acceptable safety and potential efficacy, and these studies should be expanded. There are many unapproved experimental agents under development to block TGF-β activity including antisense oligonucleotides and RNA, monoclonal antibodies, ligand traps and peptides, and small-molecule inhibitors, which are in various stages of clinical development (34, 103). Further, experimental evaluation of such agents in DMD models should be undertaken.

DRUGS WITH ANTI-FIBROTIC ACTIVITY

Approved phosphodiesterase inhibitors, such as sildenafil, which reduce muscle weakness and fibrosis in mdx mouse models (104) and alleviate cardiac dysfunction (105), are being evaluated in clinical trials. This agent does not appear to exert anti-fibrotic effects through modulation of TGF-β, but instead is thought to counteract nitric oxide synthase deficiency at the myofiber membrane and improve blood flow. Pentoxifylline, a nonselective

phosphodiesterase inhibitor with both anti-inflammatory and antifibrotic activities (thought to act through modulation of TGF-β signaling) (106), nonetheless failed to prevent deterioration in muscle function over a 1-year evaluation period in DMD patients (Table 1) (107). Pirfenidone, an antifibrotic agent (108) that has been shown to inhibit the secretion of TNF-α and TGF-β and to inhibit TGF-β signaling by preventing nuclear accumulation of active Smad2/3 complexes in animal studies (109), is approved in Europe and the United States for idiopathic pulmonary fibrosis (Table 1). Such an agent could be considered for further evaluation in DMD.

CONCLUSION

In addition to providing short-term benefits akin to steroid therapy, immunomodulation in DMD could be an adjunct treatment for other therapies (such as gene and exon skipping therapies) that may provide long-term benefit. Boosting immunosuppression to both reduce inflammation and induce immune tolerance may foster conditions that increase the likelihood of success for such treatments. Such worthy goals are likely to be achieved by activating T_{regs}, which reduced inflammation and fibrosis in the mdx mouse model. Critically, T_{regs} and other anti-inflammatory approaches will be of continued importance in taming inflammation even after successful dystrophin-replacement therapy. Dystrophin-replacement efforts to date have focused on elaboration of partial dystrophin species such that chronic inflammation is likely to continue even after the successful deployment of such treatments. Finally, intervening at the earliest stages of disease, before muscle loss and fibrosis, will be critical to effect long-term disease amelioration in DMD.

Acknowledgments

K.N. is the co-founder and president of AGADA Biosciences, Halifax, Canada, and is cofounder of ReveraGen BioPharma, Maryland, USA. E.H. is the founder, the CEO, and a board member of ReveraGen BioPharma and is vice president and cofounder of AGADA Biosciences. ReveraGen BioPharma is developing an anti-inflammatory drug, VBP15, for the treatment of DMD.

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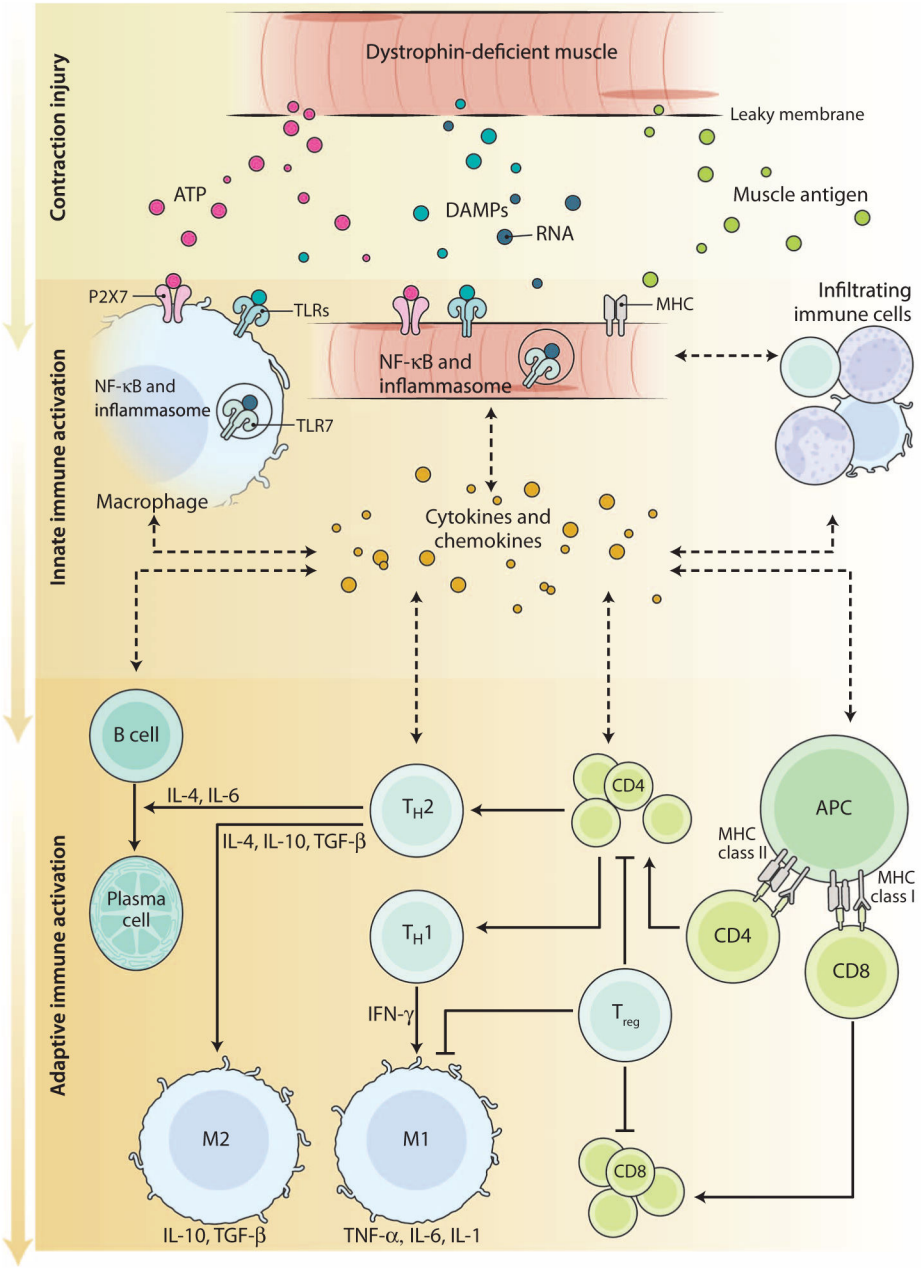

Fig. 1. Initiation and perpetuation of inflammatory responses in dystrophic muscle

Dystrophin-deficient muscle cells are susceptible to contraction-induced injury, culminating in muscle necrosis and the release of DAMP molecules including ATP (adenosine 5′-triphosphate) and nucleic acids. Released muscle proteins may serve as neoantigens. Engagement by DAMPs of TLR7 and the ionotropic receptor P2X7 on skeletal muscle cells and macrophages triggers innate immune activation and a chronic inflammatory response. Concomitantly, MHC presentation of peptides derived from muscle antigens initiates an adaptive immune response. Cytokines and chemokines released in the milieu attract and activate additional infiltrating immune cells including neutrophils and antigen presenting cells (APCs), such as M1 macrophages and dendritic cells. The APCs, in turn, activate

recruited lymphocytes ($CD4^+$ and $CD8^+$ T cells), leading to propagation of adaptive immune responses (T_H1, T_H2), which are dampened by T_{regs}. Although B cells have been detected in DMD muscle, there is no known role for B cells in antigen presentation or antibody production in the context of DMD. The balance between the T_H1 and the T_H2 adaptive immune response creates a regulatory feedback mechanism that leads to activation of either M1 or M2 macrophages, which affects the severity of muscle inflammation or the efficiency of muscle regeneration, respectively.

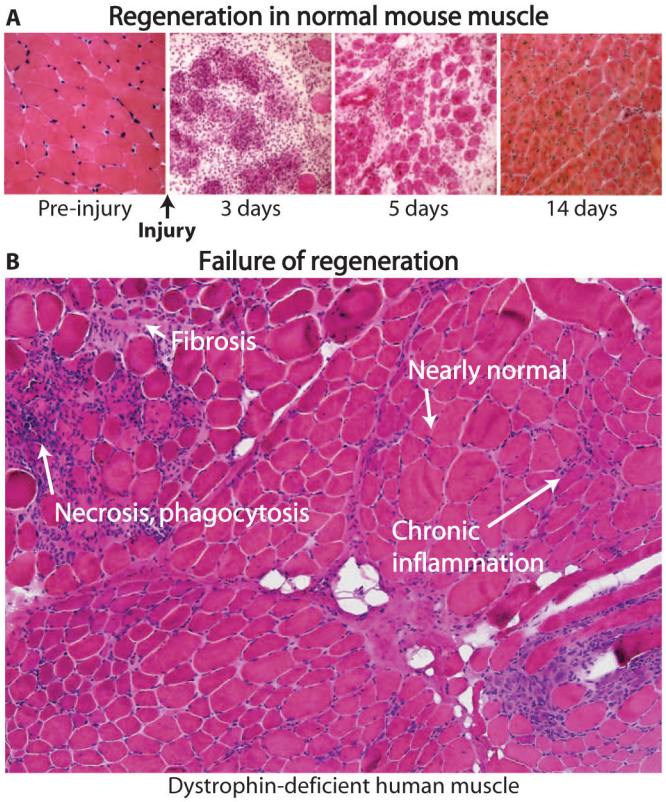

Fig. 2. Normal but not dystrophic skeletal muscle regenerates after injury

(**A**) Stages of regeneration in normal mouse muscle after injury. Regeneration of skeletal muscle in response to injury is a highly synchronized process. Within 24 hours of injury, mouse muscle becomes infiltrated with neutrophils. Within 2 to 3 days, the injured muscle is infiltrated by pro-inflammatory M1 macrophages. During days 5 to 10, the resolution and repair phases of regeneration take place and muscle is predominantly populated by remodeling M2 macrophages. M2 macrophages are essential for complete muscle regeneration, which is achieved by day 14. (**B**) Asynchronous degeneration/regeneration in human dystrophin-deficient muscle. Repair of human dystrophin-deficient muscle after injury is impaired due to asynchronous bouts of degeneration and regeneration, leading to the release of cytokines, such as TGF-β, that initiate and perpetuate fibrosis. Shown is a muscle biopsy from a DMD patient revealing regions of nearly normal myofibers; chronic inflammation (between myofibers); phagocytosis by neutrophils and macrophages, and necrosis; and fibrosis (failed regeneration).

Table 1

Immunomodulators in DMD

NFAT, nuclear factor of activated T cells; PDE, phosphodiesterase.

Drug/compound	Target	Pathological process	Preclinical trials	Clinical trials/use
		Current treatments		
Prednisone, deflazacort	NF-κB, others	Anti-inflammatory	Yes	Yes
VBP15	NF-κB, membrane protection	Anti-inflammatory, sarcolemma stability	Yes	Yes*
Cyclosporine	NFAT	Anti-inflammatory	Yes	Yes†
Azathioprine	Purine synthesis	Anti-inflammatory	Yes	Yes†
Poloxamer	Membrane protection	Sarcolemma stability	Yes	Yes‡
Gene therapy	Dystrophin replacement	Sarcolemma stability	Yes	Yes
Exon skipping	Dystrophin replacement	Sarcolemma stability	Yes	Yes
TLR7/8/9 antagonists	TLR7/8/9	Anti-inflammatory	Yes	No
NEMO peptide	NF-κB	Anti-inflammatory	Yes	No
Infliximab	TNF-α	Anti-inflammatory	Yes	No
IL-2/anti–IL-2 complex	T_{regs}	Anti-inflammatory	Yes	No
Pentoxifylline	PDE inhibitor	Anti-fibrotic	Yes	Yes
Pirfenidone	TGF-β signaling	Anti-fibrotic	Yes	No
Losartan	Angiotensin type 1 receptor inhibitor	Anti-fibrotic	Yes	Yes
Lisinopril	Angiotensin-converting enzyme inhibitor	Anti-fibrotic	Yes	Yes
Anti–IL-6	IL-6	Anti-inflammatory	Yes	No
Anti-myostatin antibodies	Myostatin	Anti-fibrotic, hypertrophy	Yes	Yes
Cromolyn	Mast cells	Membrane stability	Yes	No
		Future options		
Chloroquine	Lysosomal pH	Anti-inflammatory	No	No
Eculizumab	Complement C5	Anti-inflammatory	No	No
Rapamycin	T_{regs} +Akt/mTOR	Anti-inflammatory, regeneration	Yes	No
Plerixafor	CXCR4	Anti-inflammatory	No	No
IL-10	Alternatively activated macrophages	Anti-inflammatory	No	No
Anti-osteopontin antibodies	Osteopontin	Anti-inflammatory, anti-fibrotic	No	No
Candesartan	Angiotensin type 2 receptor inhibitor	Anti-fibrotic	No	No

*Phase 1, 2015.

†Not effective.

‡Effective in heart.