

# Anti-inflammatory drugs for Duchenne muscular dystrophy: focus on skeletal muscle-releasing factors

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**Abstract:** Duchenne muscular dystrophy (DMD), an incurable and a progressive muscle wasting disease, is caused by the absence of dystrophin protein, leading to recurrent muscle fiber damage during contraction. The inflammatory response to fiber damage is a compelling candidate mechanism for disease exacerbation. The only established pharmacological treatment for DMD is corticosteroids to suppress muscle inflammation, however this treatment is limited by its insufficient therapeutic efficacy and considerable side effects. Recent reports show the therapeutic potential of inhibiting or enhancing pro- or anti-inflammatory factors released from DMD skeletal muscles, resulting in significant recovery from muscle atrophy and dysfunction. We discuss and review the recent findings of DMD inflammation and opportunities for drug development targeting specific releasing factors from skeletal muscles. It has been speculated that nonsteroidal anti-inflammatory drugs targeting specific inflammatory factors are more effective and have less side effects for DMD compared with steroidal drugs. For example, calcium channels, reactive oxygen species, and nuclear factor- $\kappa$ B signaling factors are the most promising targets as master regulators of inflammatory response in DMD skeletal muscles. If they are combined with an oligonucleotide-based exon skipping therapy to restore dystrophin expression, the anti-inflammatory drug therapies may address the present therapeutic limitation of low efficiency for DMD.

**Keywords:** calcium channels, ryanodine receptor 1, exon skipping, NF- $\kappa$ B, myokine, ROS

## Introduction

Duchenne muscular dystrophy (DMD), the most common form of muscular dystrophy, involves progressive deterioration of muscle function, affecting up to one in 3,800–6,000 live male births.<sup>1</sup> DMD is caused mainly by a frameshift deletion, nonsense, or duplication mutations in the DMD gene on the X chromosome (Xp21.2), which encodes the protein dystrophin.<sup>2</sup> Dystrophin is a member of the spectrin superfamily of cytoskeletal proteins. Its full-length mRNA is mainly expressed in skeletal and cardiac muscles, as well as in small amounts in brain. In healthy muscle, dystrophin is located on the intracellular surface of the sarcolemma, along with the sarcomeres.<sup>3</sup> The structure is called the costamere, and it constitutes the structural cornerstone of muscular cells. At costameres, dystrophin assembles with the dystrophin-associated glycoprotein complex, which stabilizes the sarcolemma during muscular contractions. Dystrophin acts as a bridging and anchoring protein by binding to F-actin through its cytoplasmic N-terminal domain and to  $\beta$ -dystroglycan through its extracellular C-terminal domain.<sup>4,5</sup> The loss of dystrophin disrupts the dystrophin-associated glycoprotein complex and

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causes membrane instability making it susceptible to damage and myofiber necrosis. Such circumstances provoke an abnormal persistence of inflammatory macrophages in the muscle, inducing chronic inflammation with impaired regeneration and ultimately fibrosis associated with the replacement of muscle by connective tissue, and consequently severe muscle dysfunction.<sup>6-8</sup> Eventually, the loss of dystrophin results in severe muscle atrophy, respiratory and cardiac failure, and death before the age of 30 years.<sup>9</sup> Despite these findings, the molecular mechanisms initiating and perpetuating inflammation in DMD are poorly understood.

The most promising therapeutic candidate to overcome the deletion of dystrophin in DMD is exon-skipping therapy using antisense oligonucleotides (ASOs). ASOs can switch splicing patterns by targeting specific sequences of pre-mRNA elements involved in exon recognition and/or consensus splice sites in a sequence-specific manner.<sup>10</sup> Targeting splice sites or putative exon splicing enhancers with ASOs can induce the removal of exons from the mature DMD transcript so that a nonsense mutation is bypassed, or alternately removal of exons around a genomic deletion can restore the mRNA reading frame. ASOs have been extensively tested in disease models and are currently being evaluated in several clinical trials including ASO-based exon53 skipping therapy at our institute in Japan (<http://www.ClinicalTrials.gov;NCT02081625>).<sup>11,12</sup>

However, each ASO has a specific antisense sequence for single exon in the *DMD* gene and is considered a new drug; therefore, ASOs for separate exons have to undergo expensive and lengthy clinical trial stages. Furthermore, challenges involving exon skipping for duplications exist given the necessity to skip the only duplicated exon, in addition to the lack of an original exon for in-frame restoration and also the inability to recruit sufficient patients for clinical trials for exon skipping for rare mutations in DMD.<sup>13</sup> Thus, in addition to the dystrophin restoration, an efficient treatment should consider the possibility of inhibiting the muscle inflammation associated with dystrophin deletion that is common to all DMD patients.

Regarding skeletal muscle inflammation, the functions of skeletal muscle as a secretory organ should be considered. The cytokines released by skeletal muscle are called “myokines”; they are considered as autocrine and paracrine factors and are important mediators of communication between skeletal muscle and other organs in the endocrine system. They can exert profound effects on glucose and lipid metabolism and can be important mediators in inflammatory processes. As such, they are involved in energy homeostasis and the pathogenesis of obesity, diabetes, and other diseases.<sup>14</sup>

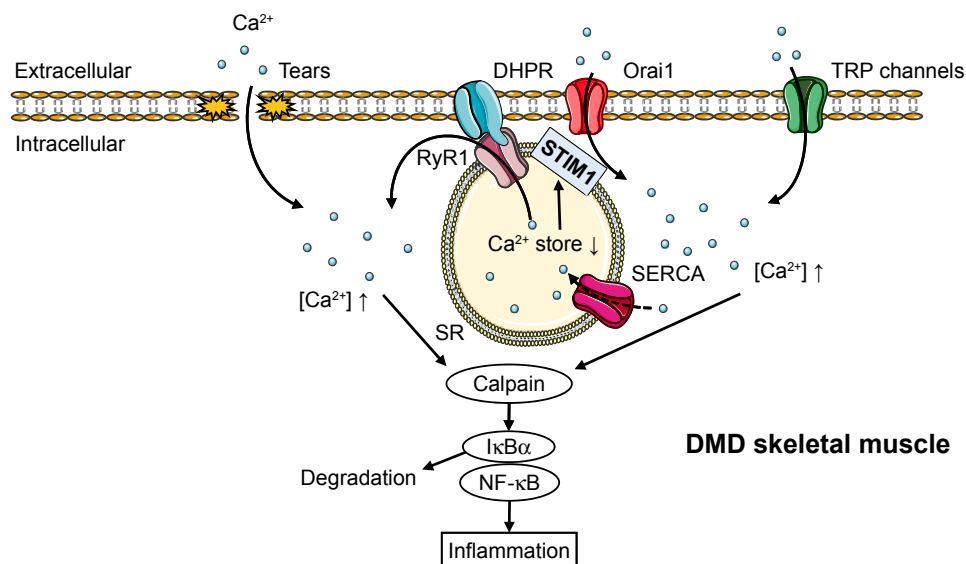
In this review, the recent findings on the inflammatory mechanisms in DMD are discussed, especially focusing on inflammatory factors released from skeletal muscle, which are regulated by calcium influx, reactive oxygen species (ROS), and nuclear factor-kappa B (NF- $\kappa$ B) (Figure 1). This review emphasizes the future directions in DMD therapy targeting those master regulators of inflammation as well as each releasing factor regulated in DMD muscles.

## **Broad anti-inflammatory drug – corticosteroids and nonsteroidal anti-inflammatory drugs targeting cyclooxygenases**

The only currently accepted pharmacological therapy for DMD is corticosteroid-based anti-inflammatory treatment. In short-term clinical trials, corticosteroids have been shown to improve muscle strength and function without clinically severe adverse effects.<sup>15,16</sup> In addition, nonrandomized trials have been shown significant beneficial effects on ambulation and cardiac function, delayed onset of both scoliosis and respiratory dysfunction, and a general amelioration of quality of life in treatments with prednisone or deflazacort for >2 years.<sup>17</sup>

However, tolerance to chronic use and heterogeneous response to treatment are well-known drawbacks of corticosteroid therapy.<sup>18</sup> Glucocorticoids act through multiple mechanisms of action, making it unclear and controversial which molecular pathways provide the efficacy in DMD treatment and which pathways are responsible for detrimental effects. For example, glucocorticoids have a side effect of impaired growth in children with asthma,<sup>19,20</sup> but the related limited muscle workload and delayed muscle maturation have been proposed to contribute to efficacy in DMD.<sup>21</sup>

Like corticosteroids, treatment with commonly used nonsteroidal anti-inflammatory drugs (NSAIDs) broadly inhibiting cyclooxygenase (COX) enzymes had only partial therapeutic effects in an animal study. COXs generate prostaglandins and lipid autacoids from arachidonic acid, leading to pathogenic mechanisms, including the inflammatory response.<sup>22</sup> Daily treatment with NSAIDs (COX inhibitors) such as aspirin and ibuprofen was effective in ameliorating muscle morphology and reducing macrophage infiltration and necrosis but did not modify the percentage of regenerating myofibers. In addition, isometric tension did not differ in treated and untreated muscles; however, resistance to fatigue decreased by treatment with aspirin and not with ibuprofen.<sup>23</sup> In this study, parecoxib, a COX-2-selective inhibitor,



**Figure 1** The hypothetical mechanism of  $\text{Ca}^{2+}$  overflow-induced inflammation response in DMD muscles.

**Notes:** There are four pathways for cytosolic  $\text{Ca}^{2+}$  increase in DMD muscles. First, membrane tears promoted in the dystrophin-deficient muscles allow direct  $\text{Ca}^{2+}$  entry by disrupted plasma membrane. Second, overactivated “leaky” RyR1 releases  $\text{Ca}^{2+}$  from SR to cytoplasm. In healthy subjects, membrane depolarization in EC coupling causes the voltage-sensitive DHPR activation, subsequent opening of RyR1 and transient  $\text{Ca}^{2+}$  release into cytoplasm, followed by  $\text{Ca}^{2+}$  reuptake into SR lumen by SERCA. On the other hand, in DMD patients, overactivated RyR1 releases  $\text{Ca}^{2+}$  into cytoplasm constitutively. Third, the depletion of ER/SR  $\text{Ca}^{2+}$  store promotes the translocation of STIM1 in ER/SR membrane to regions close to the plasma membrane, where STIM1 activates Orai1 overexpressed in DMD, allowing  $\text{Ca}^{2+}$  influx through plasma membrane into cytosol. Fourth, overexpressed TRP calcium channels on plasma membrane in DMD induce cytosolic  $\text{Ca}^{2+}$  increase from extracellular. The upregulated cytoplasmic  $\text{Ca}^{2+}$  leads to calpain overactivation, inducing NF- $\kappa$ B activation mediated by I $\kappa$ B $\alpha$  degradation, and eventually stimulating inflammatory cytokines production and release.

**Abbreviations:** DHPR, dihydropyridine receptor; DMD, Duchenne muscular dystrophy; EC, excitation-contraction; ER, endoplasmic reticulum; NF- $\kappa$ B, nuclear factor-kappa B; RyR, ryanodine receptor; SERCA, sarcoplasmic/endoplasmic-reticulum  $\text{Ca}^{2+}$ -ATPase; SR, sarcoplasmic reticulum; STIM, stromal interaction molecule; TRP, transient receptor potential.

was ineffective against DMD pathogenesis. COX-2 is known to be activated mainly in leukocytes, not muscles. In addition, steroids showed robust inhibition of COX-2 in leukocytes, which had only partial effects as mentioned earlier.<sup>24</sup> Therefore, it is possible that in order to treat DMD efficiently, the inflammatory process needs to be inhibited not only in leukocytes but in muscles as well.

Considering how problematic and insufficient corticosteroids and NSAIDs appear to be as anti-inflammatory agents in the treatment of DMD, it is possible that more specific, safer, and more effective drugs need to be developed in this area.

## Potential drugs targeting inflammatory mechanisms in DMD

### Store-operated $\text{Ca}^{2+}$ entry and transient receptor potential calcium channels

Based on the mechanism of inflammation in DMD, initial targets for anti-inflammatory drugs are calcium channels. Loss of dystrophin leads to a structurally weaker plasma membrane that is easily damaged during muscle contraction and allows both extracellular calcium influx and release of endogenous ligands. The upregulated cytoplasmic  $\text{Ca}^{2+}$  leads to calpain overactivation in DMD, which can activate the

NF- $\kappa$ B inflammatory pathways mediated by degradation of I $\kappa$ B $\alpha$  (an NF- $\kappa$ B inhibitor)<sup>25</sup> and stimulate inflammatory cytokine release, which further impairs muscle regeneration (Figure 1). This pathway induces abnormal persistence of inflammatory cells such as macrophages.<sup>26,27</sup> In fact, elevated  $\text{Ca}^{2+}$  influx through calcium channels was sufficient to induce a dystrophic phenotype, including increased central nucleated myofibers, fibrosis, and infiltration of inflammatory cells<sup>28</sup> (Figure 1). However, the mechanism of calcium over-influx in DMD muscle is not clearly understood. One of the proposed models suggest that extracellular calcium leaks into dystrophin-deficient myofibers during muscle contraction, inducing local hypercontraction after repeated contraction, which magnifies membrane damage on adjacent lengthened regions in the same or adjacent muscle fibers.<sup>29</sup> In contrast, dystrophin deficiency increases calcium concentration mediated by 1) store-operated  $\text{Ca}^{2+}$  entry (SOCE) machinery as a result of by reduction of intracellular  $\text{Ca}^{2+}$  stores, 2) concentrated stretch-activated channels on plasma membrane such as the transient receptor potential (TRP) cation channel, and 3) the oxidized, overactivated “leaky” ryanodine receptor 1 (RyR1) described in “ROS and ryanodine receptor 1” section (Figure 1).

The depletion of endoplasmic/sarcoplasmic reticulum (ER/SR)  $\text{Ca}^{2+}$  stores promotes the translocation of stromal interaction molecule 1 (STIM1), a calcium sensor in the ER/SR membrane, to regions close to the plasma membrane, where STIM1 activates Orai, a pore-forming unit that allows  $\text{Ca}^{2+}$  influx through the plasma membrane into the cytosol.<sup>30</sup> The expression of Orai1 was elevated in the dystrophic muscles, whereas STIM1 levels remained largely unchanged, together with increased SOCE activity in adult muscles of mdx mice. When Orai1 was inhibited with BTP-2, a specific SOCE inhibitor injected for 2 weeks in mdx mice, the cytosolic calpain1 activity in myofibers was significantly reduced, indicating that upregulation of Orai1-mediated SOCE pathway contributed to the disrupted  $\text{Ca}^{2+}$  homeostasis in mdx muscle.<sup>30</sup>

TRP channels promote calcium overload from extracellular calcium as a result of their expression in plasma membrane. In skeletal muscle, several isoforms of the TRPC (canonical), TRPV (vanilloid), and TRPM (melastatin) subfamilies are expressed. In particular, TRPC1, C3, and C6; TRPV1, V2, and V4; and TRPM4 and M7 have been consistently found in cultured myoblasts or in adult muscles; however, only some of the TRPC and TRPV isoforms have been studied in skeletal muscle.<sup>31</sup>

In the TRPC subfamily, expression of TRPC1, C3, and C6 was increased in dystrophic muscle.<sup>30,32</sup> The abnormal activation of these channels in mdx fibers might be mediated by ROS production and Src kinase activation.<sup>32</sup> TRPC1 may contribute through binding with STIM1–Orai1 complex as a result of  $\text{Ca}^{2+}$  store depletion.<sup>31</sup> Additionally,  $\text{Ca}^{2+}$  influx

through TRPC3 and C6 channels has been shown to be sufficient to induce muscle dystrophy.<sup>28</sup>

In the TRPV subfamily, Iwata et al<sup>33</sup> reported that TRPV2 was abundantly expressed in the sarcolemma of dystrophic myocytes and that cyclic cellular stretch increased TRPV2 translocation to the membrane. It was found that dominant-negative inhibition of endogenous TRPV2 in mdx mice suppressed the calcium increase in muscle fibers and eased dystrophic pathology, that is, the increased number of central nucleus and fiber size variability/fibrosis/apoptosis, elevated serum creatine kinase levels, and reduced muscle performance.<sup>33</sup> Tranilast, a clinically used antiallergic drug, is also known to be a TRPV2 blocker experimentally,<sup>34</sup> and its administration was found to be efficacious in reducing serum creatine kinase levels in mdx mice<sup>35</sup> and fibrosis in mdx skeletal muscles, leading to improved resistance to muscle fatigue.<sup>36</sup>

Thus far, the possible involvement of TRPV4 in DMD has not been investigated; however, it is notable that TRPV4 was activated by phospholipase A2,<sup>37</sup> an enzyme involved in SOCE in dystrophic myofibers,<sup>38</sup> and that its expression is increased by 10- to 40-fold in dystrophinopathies<sup>39</sup> (Table 1).

## ROS and ryanodine receptor I

In addition to calcium influx, elevated ROS production in DMD is a potential target for anti-inflammatory therapy, as suggested by the reports showing the inhibitory effect of the antioxidant N-acetylcysteine on muscle damage in mdx mice accompanied with reduced NF- $\kappa$ B activity<sup>40</sup> and also the contribution of ROS to RyR1 or TRPV1-induced intracellular  $\text{Ca}^{2+}$  increase.<sup>41,42</sup>

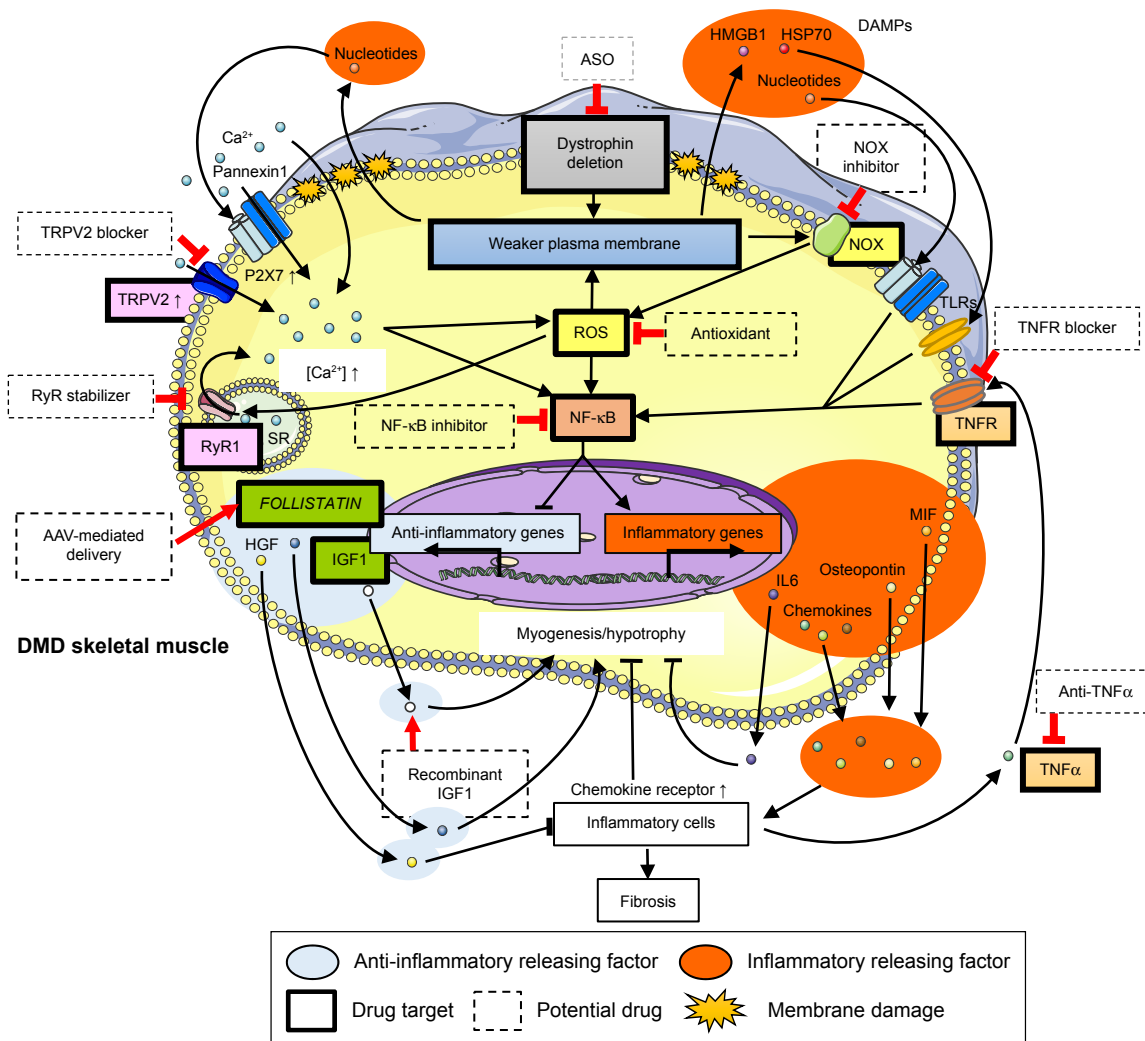
**Table 1** Regulation of  $\text{Ca}^{2+}$  transport-related proteins in DMD muscles

<b>Ca<sup>2+</sup> transport-related proteins</b>	<b>Regulation in DMD muscles</b>	<b>Role in Ca<sup>2+</sup> transport</b>	<b>Activator</b>	<b>References</b>
<b>SOCE machinery</b>				
STIM	→	Calcium sensor in ER/SR membrane and activator of Orai1	Depletion of $\text{Ca}^{2+}$ stores in ER/SR	30
Orai1	↑	Calcium channel in plasma membrane		
<b>TRPC channels</b>				
TRPC1	↑	Calcium channels in plasma membrane	ROS production and Src kinase activation	30, 32
TRPC3	↑			
TRPC6	↑			
<b>TRPV channels</b>				
TRPV1	Unknown	Calcium channels in plasma membrane	ROS production Stretch of plasma membrane Phospholipase A2	33
TRPV2	↑			
TRPV4	Unknown			
<b>Ryanodine receptor</b>				
RyR1	↑	Calcium channel in SR	ROS production	41

**Abbreviations:** DMD, Duchenne muscular dystrophy; ER, endoplasmic reticulum; ROS, reactive oxygen species; RyR, ryanodine receptor; SOCE, store-operated  $\text{Ca}^{2+}$  entry; SR, sarcoplasmic reticulum; STIM, stromal interaction molecule; TRP, transient receptor potential; TRPC, TRP canonical; TRPV, TRP vanilloid.

The mechanical distension of the sarcolemma induced by contraction causes the formation of superoxide anion radicals by the action of nicotinamide adenine dinucleotide phosphate oxidases (NOX), including NOX type 2 and type 4 in skeletal muscle. This radical cannot be scavenged by nitric oxide to form peroxynitrite because of its low presence in DMD

muscles, followed by the oxidization of RyRs and indirect activation of stretch-activated channels<sup>41</sup> (Figure 2). Notably, NOX inhibition with diapocynin, a dimer of the commonly used NOX inhibitor apocynin, was shown to inhibit the loss of strength of skeletal muscles induced by eccentric contractions in mdx mice, a model of DMD, to levels similar to those



**Figure 2** Overview of inflammatory mechanism in DMD muscles and related potential drugs.

**Notes:** The lack of dystrophin stabilizing sarcolemma leads to weaker plasma membrane susceptibility of muscle contraction-induced damage, followed by both extracellular calcium influx and release of DAMPs, as well as NOX-induced ROS production. ROS contributes further membrane permeability through peroxidation of sarcolemmal lipids. Nucleotides such as ATP in DAMPs also induce pannexin1 channel-induced calcium influx triggered by upregulated P2X7 receptor in DMD muscles. Other mediators of calcium upregulation are concentrated stretch-activated channels like TRPV2 and oxidized leaky RyR1 in the membrane of the sarcoplasmic reticulum. Upregulated cytoplasmic calcium induces additional ROS production from mitochondria. Calcium influx, ROS production, and signaling from P2X7 and TLRs, receptors for DAMPs induce NF-κB activation, a major transcription factor for DMD inflammation contributing to enhanced expression of inflammatory genes and downregulated expression of anti-inflammatory genes. Of the inflammatory cytokines, chemoattractants such as chemokines, MIF, and osteopontin bind to their receptor on inflammatory cells and attract them into DMD skeletal muscles, which are important mediators of chronic inflammation resulting in suppressed muscle regeneration and promoted fibrosis. Inflammatory macrophages in DMD secrete TNF-α inducing positive feedback of NF-κB activation mediated by TNFR. Of the anti-inflammatory genes that were inhibited by NF-κB, HGF suppresses the infiltration of inflammatory cells and IGF-1 induces myogenesis and muscle hypertrophy, the counterparts of which include IL6 and myostatin that are upregulated in DMD muscles. Additionally, secreted follistatin promotes muscle hypertrophy as an antagonist of TGF-β family members including myostatin. In addition to those DMD inflammatory pathways, this schematic shows the potential drugs targeting indicated specific molecules. The up arrow next to each receptor indicates upregulation in DMD skeletal muscles.

**Abbreviations:** ASO, antisense oligonucleotide; DAMPs, danger-associated molecular patterns; DMD, Duchenne muscular dystrophy; HGF, hepatocyte growth factor; HMGB, high-mobility group box protein; HSP, heat shock protein; IGF, insulin-like growth factor; IL, interleukin; MIF, macrophage migration inhibitory factor; NF-κB, nuclear factor-kappa B; NOX, nicotinamide adenine dinucleotide phosphate oxidase; ROS, reactive oxygen species; RyR, ryanodine receptor; SR, sarcoplasmic reticulum; TLR, Toll-like receptor; TNF, tumor necrosis factor; TNFR, TNF receptor; TRPV, transient receptor potential vanilloid.

in wild-type mice.<sup>43</sup> In a potential therapy targeting downstream of ROS production, stabilization of the closed state of RyRs by Rycals, a RyR channel inhibitor, restored cardiac and skeletal muscle function by normalizing oxidized and overactivated RyRs.<sup>44,45</sup>

In contrast, Ca<sup>2+</sup> overload in mitochondria directly increases ROS levels,<sup>46</sup> which further contributes to membrane permeability through sarcolemmal lipid peroxidation.<sup>47</sup> The antioxidant idebenone (Catena<sup>®</sup>; Santhera Pharmaceuticals, Liestal, Switzerland), a synthetic analog of ubiquinone (coenzyme Q10), is currently under clinical trials to test its efficacy in the prevention of skeletal muscle function loss and heart pump function reduction. Santhera Pharmaceuticals has confirmed the safety of idebenone in DMD patients (<https://ClinicalTrials.gov/ct2/show/NCT00654784>). There is an ongoing Phase II extension trial to test its safety and efficacy in the long-term. A Phase III trial was also carried out to assess the effect of idebenone on pulmonary function, motor function, muscle strength, and quality of life in patients not treated with corticosteroids. The results show that idebenone was well tolerated, and a slower decline in respiratory function was observed for treated patients compared to that observed for the placebo group (<https://ClinicalTrials.gov/ct2/show/NCT01027884>). These beneficial effects of idebenone can be explained by its ability to protect against mitochondrial respiratory chain dysfunction and reduce ROS production.<sup>48</sup>

Santhera plans to file for marketing authorization in 2016. Furthermore, it is planning a placebo-controlled Phase III study in corticosteroids-treated patients. Additionally, pre-clinical studies with green tea extract and its active ingredient epigallocatechin gallate revealed histological and functional improvement in mdx mice.<sup>49,50</sup> Furthermore, early treatment with green tea extract reduced dystrophic muscle pathology, potentially by regulating NF-κB activity in regenerating fibers.<sup>51</sup> Epigallocatechin gallate for DMD patients is now under Phase II/III trials at Charité University Hospital Berlin, Germany (<https://www.ClinicalTrials.gov/ct2/show/NCT01183767>). Flavocoxid is another antioxidant that has been tested in a Phase I trial in DMD patients to assess its safety (<https://www.ClinicalTrials.gov/ct2/show/CAT-1004>) (Table 2; Figure 2).

## NF-κB, tumor necrosis factor-α, and chemokines

NF-κB, one of the major transcription factor regulating inflammatory pathways, is also an important target for anti-inflammatory therapy. CAT-1004, an inhibitor of NF-κB has

**Table 2** The potential drugs for DMD anti-inflammatory therapy

Target	Potential drug	Company/sponsor	Mode of action	Clinical status	Reference*
Calcium channel	TRPV2	Kissei Pharmaceuticals	TRPV2 blocker	Clinically used anti-allergic drug	
	RyR1	ARMGO Pharma	Stabilizes the closed state of RyRs and inhibits calcium release from SR	Phase I	
ROS	Idebenone	Santhera Pharmaceuticals	Antioxidant	Phase III completed, preparation for marketing authorization	NCT01027884
	EGCg	Charite University Hospital Berlin	Antioxidant	Phase II/III	NCT01183767
NF-κB	Flavocoxid	University of Messina	Antioxidant	Phase I completed	NCT0133529
	CAT-1004	Catabasis	NF-κB inhibitor	Phase I/II	NCT02439216
	VBPI5	ReveraGen Biopharma	NF-κB inhibitor, membrane stabilizer	Phase I	NCT02415439
	Remicade	Merck/Schering-Plough	Antibody to human TNF-α	Preclinical	
Follistatin	Etanercept	Wyeth	Blocks soluble receptor to TNF-α	Preclinical	
	AAV.CMV.FS344	Nationwide Children's Hospital	Delivery of follistatin using adeno-associated virus	Phase I/II	NCT02354781
IGF-I	INCRELEX	Children's Hospital Medical Center	Recombinant IGF-I	Phase II	NCT01207908

**Note:** \*[ClinicalTrials.gov](https://ClinicalTrials.gov) identifier.

**Abbreviations:** DMD, Duchenne muscular dystrophy; EGCg, epigallocatechin gallate; IGF, insulin-like growth factor; NF-κB, nuclear factor-kappa B; NOX, nicotinamide adenine dinucleotide phosphate oxidase; ROS, reactive oxygen species; RyR, ryanodine receptor; TNF, tumor necrosis factor; TRPV, transient receptor potential vanilloid.

completed Phase I clinical trial (<https://ClinicalTrials.gov/ct2/show/VBP15>) (Table 2).

NF- $\kappa$ B is activated in dystrophic muscles, possibly following calcium influx and/or ROS production,<sup>25,40</sup> and targets a wide range of genes, including inflammatory cytokines and chemokines.<sup>52</sup> Interestingly, NF- $\kappa$ B is activated among the earliest histological features of DMD neonates,<sup>53–55</sup> years before symptoms appear. It suggests that very early treatment of DMD patients with NF- $\kappa$ B suppression therapy may prevent or delay the onset of muscle dysfunction as a result of improved muscle regeneration and reduction of fibrosis.

Ablation of one allele of the p65 subunit of NF- $\kappa$ B was sufficient to improve pathology in mdx mice. In addition, a study explored conditional deletion of I $\kappa$ B kinase (IKK)  $\beta$  in mdx mice, a component of the IKK complex with IKK $\alpha$  as catalytic subunits that induce NF- $\kappa$ B activation by I $\kappa$ B degradation. It was found that NF- $\kappa$ B in activated macrophages promotes inflammation and muscle necrosis in skeletal muscle fibers, resulting in limited regeneration through the inhibition of muscle progenitor cells. In addition, specific pharmacological inhibition of IKK resulted in improved pathology and muscle function in mdx mice.<sup>26</sup>

Recently, Heier et al identified VBP15, a compound structurally related to glucocorticoids with similar anti-inflammatory properties but without steroidal side effects.<sup>56</sup> VBP15 inhibited NF- $\kappa$ B-induced tumor necrosis factor (TNF)- $\alpha$  release mediated by the glucocorticoid receptor, independently of the glucocorticoid response elements (eg, classical steroid receptor transactivation or hormonal properties), activation or upregulation that is implicated in a number of glucocorticoid side effects. It also had protective physicochemical effects on the cell membrane. The translation of these drug mechanisms into mdx mice improved muscle strength, live imaging, and pathology through both preventive and post-onset intervention regimens. VBP15 is now under a Phase I clinical trial (Table 2).

Furthermore, the inhibitors of TNF- $\alpha$ , a chemokine that induces NF- $\kappa$ B activation, have shown some potential in improving DMD pathology in animal studies. Remicade, a human anti-TNF- $\alpha$  antibody and etanercept, a blocker of soluble TNF- $\alpha$  receptor, are two candidates clinically used to treat inflammatory disorders (Table 2). Remicade delayed and reduced the inflammation and disruption of dystrophic muscle without adverse effects in mdx mice.<sup>57</sup> Etanercept inhibited exercise-induced force reduction in skeletal muscle<sup>58</sup> and protected against exercise-induced myofiber necrosis in mdx mice.<sup>59</sup> Furthermore, a murine-specific

TNF- $\alpha$  antibody significantly reduced contractile dysfunction and myofiber necrosis.<sup>60</sup>

Chemokines, whose expression is regulated by NF- $\kappa$ B, are major contributors to DMD pathogenesis promoting persistent inflammatory cells such as CD4<sup>+</sup> and CD8<sup>+</sup> T-cells, neutrophils, eosinophils, and inflammatory macrophages.<sup>27</sup> Besides immune cells, the main source of chemokines in skeletal muscle, muscle fibers can also chemoattract myeloid cells secreting chemokines. We recently found that NF- $\kappa$ B is activated in C2C12 myotubes upon muscle contraction and upregulate C-C motif chemokine ligand (CCL) 2 secretion, inducing THP-1 monocyte chemoattraction.<sup>61</sup> In addition, several CC chemokines including CCL2, which are the components of the largest family of chemokines, and chemoattractive for diverse inflammatory cells, showed increased expression in mdx muscle.<sup>62</sup> These findings suggest that increased CCL2 secretion from DMD muscles may also contribute to monocyte chemoattraction into skeletal muscles. Furthermore, CCL receptor (CCR) 2 is the only CCL2 receptor whose expression was upregulated in muscles of mdx mice.<sup>63</sup> CCR2 is expressed highly in Ly6C<sup>high</sup> inflammatory monocytes, which polarize CD11b<sup>high</sup> inflammatory macrophages. CCR2 deficiency in mdx mice preferentially reduced the CD11b<sup>high</sup> population of macrophages and promoted the recovery of normal macrophage polarization characteristics. Consequently, these mice improved characteristic histopathological features of the disease and increased the force-generating capacity of the diaphragm.<sup>6</sup>

Also, C-X-C motif chemokine ligands (CXCL) such as CXCL1, CXCL2, CXCL3, CXCL8, and CXCL11 for neutrophils contributing to the inflammatory phase during regeneration and absent from normal muscle fibers were induced in DMD myofibers.<sup>62</sup> In addition, we found the secretion of macrophage migration inhibitory factor (MIF), an inflammatory cytokine and chemoattractant, was regulated by contraction in C2C12 myotubes.<sup>64</sup> MIF is known to be expressed mainly in leukocytes. However, in an immunohistochemical study, MIF was detected not only in immune cells but also in muscle fiber membrane areas of infiltration, necrosis, myophagocytosis, degeneration, and regeneration in muscular dystrophy samples.<sup>65</sup> MIF binds to the CXC chemokine receptors CXCR2 and CXCR4. CXCR2 is the receptor for CXCL1, 2, 3, and 8, which are the CXCLs upregulated in DMD muscles, and CXCR4 is the receptor for CXCL12, which is also upregulated in regenerating myofibers from DMD patients<sup>62</sup> and thus can contribute to the inflammation. However, CXCL12 and CXCR4 may be difficult to use as candidates for anti-inflammation therapy because of their

major role in muscle regeneration.<sup>66</sup> Table 3 describes the potential of chemoattractants and these receptors as future therapeutic targets for DMD.

A second mechanism by which IKK/NF- $\kappa$ B signaling regulates the dystrophic process is inhibition of muscle regeneration. Muscle regeneration after injury represents a coordinated sequence of events from the activation of quiescent satellite cells into myoblasts to their subsequent fusion to newly formed myotubes.<sup>67</sup> NF- $\kappa$ B has been previously shown to inhibit the differentiation sequence by MyoD repression<sup>68</sup> and by preventing myoblast fusion *in vitro*.<sup>69</sup> Additionally, an *in vivo* study showed that TNF- $\alpha$ , a potent inducer of NF- $\kappa$ B signaling, inhibited myogenesis through repression of MyoD.<sup>68</sup> Genetically or pharmacological blocking of NF- $\kappa$ B function promoted the formation of new myofibers in response to degeneration.<sup>26</sup> IKK deletion in muscles in response to acute injury could also lead to improved regeneration<sup>70</sup> (Figure 2).

In addition to upregulating inflammatory cytokines, NF- $\kappa$ B suppresses hepatocyte growth factor (HGF), an anti-inflammatory cytokine. The mdx; NF- $\kappa$ B p65<sup>+/-</sup> muscle at 4 weeks of age showed significantly higher HGF expression,

correlating with reduced leukocyte infiltration and increased muscle fiber formation. The phenotypic improvements of muscle in these mice were reversed by silencing HGF preferentially in myogenic cells, resulting in significant degeneration of the diaphragm.<sup>71</sup>

## Danger-associated molecular patterns

The disruption of plasma membrane or necrosis following calcium influx in DMD leads to the release of cytoplasmic molecules called danger-associated molecular patterns (DAMPs) that include nucleotides, high-mobility group box protein 1 (HMGB1), hyaluronic acid, biglycan, and heat shock proteins (HSPs). DAMPs can induce chronic inflammation as the ligands of Toll-like receptors (TLRs)<sup>72</sup> (Figure 2). Recent proteomic studies of DMD subjects and mdx mice revealed an increased presence of several potential TLR2 ligands in the circulation, including serum amyloid A, HSP70, and fibrinogen.<sup>73,74</sup> Other studies detected HMGB1, a shared TLR2/4 ligand, as a potential early inflammatory target in mdx mice,<sup>7</sup> in addition to increased HSP70 expression in DMD muscles,<sup>75</sup> and fibrinogen as a driver of fibrosis in dystrophic muscle<sup>76</sup> (Table 3).

**Table 3** The upregulated inflammatory factors and their receptors in DMD muscles as the future targets for anti-inflammatory drugs

Inflammatory factor (ligands)	Receptors	Upregulated ligands or receptors
<b>DAMPs</b>		
ATP	P2X7	P2X7 mRNA (muscles of mdx mice <sup>81</sup> ), P2X7 protein (dystrophic myoblasts and myotubes <sup>81,85</sup> )
Amyloid A	TLR2	Serum amyloid A-2 protein (serum from mdx mice <sup>73</sup> )
HSP70		HSP70 IA/IB protein (serum from DMD patients <sup>74</sup> ), HSP70 protein (human DMD muscles <sup>75</sup> )
Fibrinogen		Fibrinogen gamma chain (serum from mdx mice <sup>74</sup> ), fibrinogen (serum from DMD patients <sup>74</sup> ), fibrinogen protein (muscles from mdx mice and DMD patients <sup>76</sup> )
HMGB1	TLR2/4	HMGB1 protein in the cytoplasm (mdx and human DMD muscles <sup>7</sup> ), HMGB1 total protein (muscles of mdx mice <sup>7</sup> )
<b>Chemoattractants</b>		
CCL2	CCR2	CCL2 mRNA and protein (muscles of mdx mice <sup>6,63</sup> ), CCR2 mRNA (muscles of mdx mice <sup>6,63</sup> )
CCL7		CC7 mRNA (muscles of mdx mice <sup>6,63</sup> )
CCL8		CCL8 mRNA (muscles of mdx mice <sup>6,63</sup> )
CCL12		CCL12 mRNA (muscles of mdx mice <sup>6</sup> )
CXCL1	CXCR2	CXCL1 protein (muscles of mdx mice <sup>62</sup> )
CXCL2		CXCL2 protein (muscles of mdx mice <sup>62</sup> )
CXCL3		CXCL3 protein (muscles of mdx mice <sup>62</sup> )
CXCL8	CXCR1, CXCR2	CXCL8 protein (muscles of mdx mice <sup>62</sup> )
CXCL11	CXCR3	CXCL11 protein (muscles of mdx mice <sup>62</sup> )
MIF	CXCR2, CXCR4	MIF protein (muscles from Becker muscular dystrophy patients <sup>65</sup> ), CXCR4 mRNA (muscles of mdx mice <sup>62</sup> )
<b>Others</b>		
IL6	IL6R	IL6 mRNA (muscles of mdx mice <sup>91,92</sup> ), IL6 protein (serum from mdx mice <sup>90,92</sup> and DMD patients <sup>90,91</sup> )
OPN	Integrin, CD44	OPN mRNA (muscles of mdx mice <sup>78</sup> ), OPN protein (in muscles and serum of mdx mice, <sup>78</sup> and dystrophic dogs <sup>99</sup> )

**Abbreviations:** CCL, C-C motif chemokine ligand; CCR, CCL receptor; CXCL, C-X-C motif chemokine ligand; CXCR, CXCL receptor; DMD, Duchenne muscular dystrophy; DAMPs, danger-associated molecular patterns; HMGB, high-mobility group box protein; HSP, heat shock protein; IL, interleukin; IL6R, IL-6 receptor; MIF, macrophage migration inhibitory factor; OPN, osteopontin; TLR, Toll-like receptor.



These reports suggest that endogenous TLR ligands released from injured skeletal muscle may be important signals for stimulating chemokine expression in both muscle fibers and hematopoietic progenitor cells mediated by TLR-dependent inflammasome and/or NF- $\kappa$ B activation, thereby helping to attract monocytes/macrophages to the site of damaged skeletal muscle. These TLR2 ligands also affected the function of other inflammatory cell types in DMD muscles such as T lymphocytes, which play an important role in regulating macrophage function and are also implicated in the pathogenesis.<sup>77,78</sup> In fact, TLR2 deletion in mdx mice led to reduced macrophage numbers within DMD muscle during the acute inflammatory phase of the disease. The reduced macrophage infiltration within diaphragms of mdx-TLR2<sup>-/-</sup> mice was associated with a significant amelioration of DMD pathogenesis, that is, reduced necrotic injury, larger regenerated myofibers, and decreased fibrosis, all of which led to a higher force-generating capacity of the muscle. In detail, TLR2 deletion in this context not only reduced macrophage infiltration but also significantly modified macrophage polarization into iNOS<sup>+</sup>CD206<sup>+</sup>, anti-inflammatory macrophages.<sup>79</sup> Furthermore, mdx mice lacking the TLR signaling adaptor protein MyD88 showed less myofiber necrosis in the diaphragm and improved limb muscle strength at 12 months of age.<sup>80</sup> Similarly, TLR4 ablation in mdx mice resulted in significant reduction of the amount of inflammatory macrophages within the diaphragm early in the pathogenesis (6–12 weeks of age), together with improved muscle histology and strength.<sup>7</sup>

In DMD, ATP release, another major pattern in DAMPs, due to the fragility of myofibers can activate plasma membrane receptors for extracellular nucleotides termed “P2 receptors”. P2X7 was substantially upregulated in skeletal muscle from mdx mice and in myoblasts isolated from DMD patients.<sup>81–85</sup> Additionally, exposure of mdx myoblasts to extracellular ATP induced a significant increase in P2X7/pannexin1 channel-dependent Ca<sup>2+</sup> influx and release of interleukin (IL)-1 $\beta$ , suggesting that nucleotides released from dystrophic muscle can trigger inflammatory response in DMD through purinergic signaling.<sup>86</sup> It was recently reported that *in vivo* blocking of the extracellular ATP/P2X purinergic signaling pathway by periodate-oxidized ATP delayed the progression of DMD pathogenesis and ameliorated the local inflammatory response in mdx mice, including reduced leukocyte infiltration and IL-6 expression<sup>87</sup> (Table 3; Figure 2).

## IL-6

IL-6 is one of the most extensively investigated myokines, and its secretion is regulated by exercise. Additionally, it

can regulate glucose and lipid homeostasis affecting liver, adipose tissue, and skeletal muscle itself.<sup>88</sup> IL-6 expression in skeletal muscle was found to be also upregulated in obesity and to contribute to increased white adipose tissue mass.<sup>89</sup>

IL-6 is present at high levels in the circulation and muscles from DMD patients and from young mdx mice. It also follows the disease time-course in DMD patients.<sup>90–92</sup> IL-6 contributes to satellite cell proliferation<sup>93</sup> and muscle growth,<sup>94,95</sup> in addition to its major role of inducing the transition from acute neutrophil infiltration to chronic mononuclear cell infiltration.<sup>96</sup> Therefore, repeated cycles of degeneration/regeneration, induced by chronic IL-6 upregulation in DMD, might induce the exhaustion of satellite cells, leading to enhancement of the dystrophic phenotype. In fact, IL-6-overexpressing mdx mice showed an exacerbation of the dystrophic phenotype wherein increased circulating levels of IL-6 promoted muscle degeneration, inflammation, exhaustion of muscle stem cells, and accumulation of fibro/adipogenic progenitors.<sup>92</sup> Importantly, the neutralization of IL-6 activity in mdx mice using an anti-IL-6 receptor antibody resulted in increased robustness for the dystrophic muscle, impeded the chronic inflammatory response, reduced muscle necrosis, and promoted muscle differentiation and maturation. These events led to the reduction of exercise-induced fiber damage and mitigation of diminished muscle strength.<sup>91</sup> The Food and Drug Administration has approved a number of novel compounds blocking IL-6 signaling for the treatment of other inflammatory disorders in the last decade; the application of these drugs to DMD patients can be due to their anti-inflammatory properties (Table 3; Figure 2).

## Osteopontin

Osteopontin (OPN) is a secreted and chemotactic phosphoprotein that plays important roles in tissue remodeling following injury.<sup>97,98</sup> Recently, elevated serum OPN levels were found in the dystrophic dogs compared with those in the wild type just before and an hour after a cesarean section birth and at the age of 3 months. In addition, the serum OPN level was significantly correlated with the phenotypic severity of dystrophic dogs at the onset of muscle weakness. Immunohistologically, OPN was upregulated in infiltrated macrophages and developmental myosin heavy chain-positive regenerating muscle fibers in the dystrophic dogs.<sup>99</sup> It is reported that a promoter polymorphism on the *OPN* gene was associated with severity of DMD. The G allele in the locus (dominant model; 35% of subjects) inhibiting the binding of transcription factors, specificity protein-1 was associated with more rapid progression and less grip strength, suggesting OPN is a genetic modifier of DMD.<sup>100</sup>

In addition, the promoter structure of the *OPN* gene has multiple predicted steroid hormone enhancers and a NF- $\kappa$ B promoter element.<sup>101</sup>

Genetic ablation of OPN in mdx mice caused significant reduction in the amount of intramuscular neutrophils and natural killer T-like cells and an increased amount of regulatory T-cells. This anti-inflammatory state resulted in a net decrease in the amount of transforming growth factor-beta (TGF- $\beta$ ) in the later stages of DMD pathogenesis. Diminished TGF- $\beta$  levels were correlated with a marked decrease in fibrosis of both diaphragmatic and cardiac muscles. These studies identified OPN as an immunomodulator and pro-fibrotic cytokine in DMD muscle<sup>78</sup> (Table 3; Figure 2).

## Myostatin and follistatin

Myostatin, a member of TGF- $\beta$  family, is a negative regulator of skeletal muscle growth<sup>102,103</sup> and is produced predominantly in muscle and blood.<sup>102,104</sup> Its expression was significantly higher in mdx mice than in wild-type mice.<sup>105</sup> The inhibitory effect of myostatin on postnatal growth is mediated by its negative regulation of satellite cell activation, proliferation, and self-renewal<sup>106</sup> as well as myoblast proliferation and differentiation.<sup>103,107</sup>

Blockade of endogenous myostatin using blocking antibodies for 3 months resulted in increased body weight, muscle mass, muscle size, and absolute muscle strength in mdx muscle, along with decreased muscle degeneration and concentrations of serum creatine kinase.<sup>108</sup> Myostatin propeptide also ameliorated the symptoms in mdx mice.<sup>109</sup>

However, clinical studies targeting myostatin have not shown sufficient therapeutic effects. MYO-029, a human myostatin antibody,<sup>110</sup> increased the muscle mass in immunodeficient mice by ~30% in 3 months.<sup>108</sup> In contrast, a double-blind randomized clinical trial in Becker muscular dystrophy indicated that MYO-029 was safe but not efficacious.<sup>110</sup> Another myostatin inhibitor, ACE-031, a soluble activin type IIB receptor was found promising in increasing muscle mass and whole body pulling tension in mdx mice.<sup>111</sup> However, a safety-tolerability study of ACE-031 in DMD patients was prematurely terminated because of minor nosebleeds and/or gum bleeding.

Follistatin, a secreted glycoprotein and functional antagonist of the TGF- $\beta$  family, which includes myostatin, is likely to be more effective as a therapeutic target to promote muscle growth than inhibition of only myostatin, as indicated by a report that heterozygous loss of follistatin resulted in retention of reduced muscle mass in a myostatin-null background.<sup>112</sup> This implies that follistatin inhibits

other TGF family members in addition to myostatin to regulate muscle size. Delivery of the *FOLLISTATIN* gene by adeno-associated virus (AAV1. CMV.FS344) in Becker muscular dystrophy patients in a Phase I/IIa clinical study showed safety and efficacy as determined by the distance walked in a 6-minute walk test, along with improved muscle histopathology.<sup>113</sup> Based on this positive result, the same strategy for DMD is currently under a Phase I/II clinical study (<https://ClinicalTrials.gov/ct2/show/NCT02354781>) (Table 2; Figure 2).

## Insulin-like growth factor-I

Contrary to the inhibition of inflammatory response, there is also the strategy of enhancement of anti-inflammatory response and hypotrophic factor targeting by insulin-like growth factor-1 (IGF-1) in skeletal muscles. The transgenic mdx mice expressing muscle-restricted IGF-1 (mIGF-1) (mdx:mIgf<sup>+/+</sup>) exhibited increase in muscle mass by at least 40% leading to similar increases in force generation in extensor digitorum longus muscles compared with mdx mice. The diaphragms from transgenic mdx:mIgf<sup>+/+</sup> mice exhibited significant hypertrophy and hyperplasia at all ages. In addition, the enhanced IGF-1 expression significantly diminished the amount of fibrosis normally observed in diaphragms of aged mdx mice. Decreased myonecrosis was also observed in diaphragms and quadriceps from the transgenic mice compared with age-matched mdx animals. Furthermore, signaling pathways associated with muscle regeneration and protection against apoptosis were significantly elevated in the transgenic mice.<sup>114</sup> Interestingly, mIGF-1 repressed the expression and activity of MIF, HMGB1, and NF- $\kappa$ B.<sup>115</sup>

IGF-1 is currently approved for severe primary IGF deficiency by the Food and Drug Administration. A Phase II clinical trial of recombinant IGF-1 (INCRELEX<sup>TM</sup>) has been initiated in glucocorticoid-treated DMD patients to test its ability to preserve muscle function over 6 months (<https://ClinicalTrials.gov/ct2/show/NCT01207908>) (Table 2).

## Conclusion

From the point of view of inflammatory mechanisms in DMD skeletal muscles, calcium channel, ROS production, and NF- $\kappa$ B pathway are potential targets for treatment as the master regulators. In fact, RyR channel blockers, antioxidants, and NF- $\kappa$ B inhibitors are under clinical trial. Those potential drugs targeting specific molecules are promising as more effective and less toxic compared with the current corticosteroid therapy used as a broad anti-inflammatory treatment. Furthermore, those master regulators of DMD inflammation

eventually induce the release of inflammatory factors such as DAMPs and inflammatory cytokines and suppress anti-inflammatory cytokines such as HGF and IGF-1 that mediate DMD pathogenesis (Figure 1). Thus, we emphasize the need to understand the underlying mechanisms of DMD pathogenesis and the secretory functions of skeletal muscle as both contributor and healer of DMD pathogenesis in order to develop the next generation of DMD drugs.

## Future perspectives

Dystrophin restoration by exon skipping in DMD is the most promising therapy, because it directly addresses the underlying pathogenic cause. Combined with this therapy, the anti-inflammatory treatments might show improved therapeutic potential. Interestingly, there are already trials using dual exon skipping of dystrophin and myostatin pre-mRNAs using ASOs.<sup>116</sup> Although the additional or synergistic effect of this combination of dystrophin restoration and muscle growth promotion is not yet clear, this ASO-using epochal idea can be applied to the treatments targeting other inflammatory factors discussed in this review with dystrophin restoration at the same time.

Additionally, clustered regularly interspaced short palindromic repeat/Cas9-mediated genome editing was recently applied to correct the DMD gene mutation itself in the germ line of mdx mice, suggesting the potential to restore DMD protein levels.<sup>117</sup> Future advances of this technique may enable genome editing of postnatal somatic cells and correction of DMD gene mutations in the muscle tissue. The combination of this strategy with anti-inflammation drugs may cooperate to attenuate DMD pathogenesis in the future.

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## Disclosure

The authors report no conflicts of interest in this work.

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