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Original Article

# Functional and Morphological Improvement of Dystrophic Muscle by Interleukin 6 Receptor Blockade

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ABSTRACT

The anti-inflammatory agents glucocorticoids (GC) are the only available treatment for Duchenne muscular dystrophy (DMD). However, long-term GC treatment causes muscle atrophy and wasting. Thus, targeting specific mediator of inflammatory response may be more specific, more efficacious, and with fewer side effects. The pro-inflammatory cytokine interleukin (IL) 6 is overproduced in patients with DMD and in the muscle of mdx, the animal model for human DMD. We tested the ability of inhibition of IL6 activity, using an interleukin-6 receptor (Il6r) neutralizing antibody, to ameliorate the dystrophic phenotype. Blockade of endogenous Il6r conferred on dystrophic muscle resistance to degeneration and alleviated both morphological and functional consequences of the primary genetic defect. Pharmacological inhibition of IL6 activity leaded to changes in the dystrophic muscle environment, favoring anti-inflammatory responses and improvement in muscle repair. This resulted in a functional homeostatic maintenance of dystrophic muscle.
These data provide an alternative pharmacological strategy for treatment of DMD and circumvent the major problems associated with conventional therapy.


## 1. Introduction

Duchenne muscular dystrophy (DMD) is an X-linked genetic disease caused by mutations in the dystrophin gene that result in dysfunctional or absent dystrophin protein. The absence of dystrophin causes instability of the dystrophin–glycoprotein complex (DGC) with alterations in intracellular signaling leading to an imbalance between protein synthesis and protein degradation, with subsequent necrosis and fibrosis (Glass, 2007). The mdx mouse strain, with a point mutation within its *dystrophin* gene, has served as the animal model for human DMD (Carnwath and Shotton, 1987).

Although the mdx mouse presents some limitations compared to DMD patients, due to the fact that skeletal muscles of mdx mice undergo extensive necrosis only early in neonatal life, it remains an ideal model for preclinical tests and proof-of-concept studies (Bentzinger et al., 2014; Bogdanovich et al., 2002; Colussi et al., 2008; Consalvi et al., 2013; Denti et al., 2006; Grounds et al., 2008; Jiang et al., 2014; Kainulainen et al., 2015; Minetti et al., 2006; Price et al., 2014; Stupka et al., 2006; Tedesco et al., 2011; Vidal et al., 2012; Villalta et al., 2011, 2014; von Maltzahn et al., 2012; Willmann et al., 2009). Different animal models, including the double mutant mice like mdx deficient for *MyoD1*, *utrophin*, *parvalbumin*, *alpha7 integrin*, or mTR (telomerase), have been generated and all of them share many phenotypical hallmarks with DMD (reviewed in Willmann et al., 2009). However, several aspects, including the genetic basis of the disease, must be considered when evaluating the animal models for use in the preclinical testing of potential new treatment options (Willmann et al., 2009). Thus, the mdx mouse, lacking a functional dystrophin gene, represents the most valid pre-clinical model, considering also that the double mutant mice do not resemble the genetic background of DMD patients and are therefore less appropriate to predict therapeutic effects (Willmann et al., 2009).

There is an acute onset of pathology (increased myofiber necrosis and elevated blood CK) around 3 weeks of age, in which mdx mice display muscle weakness similarly to DMD patients (Grounds et al., 2008) and the mdx muscles appear more susceptible to fatigue in vivo than control mice, similarly to other dystrophic models (Willmann et al., 2009).

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Among factors involved in the pathogenesis of muscular dystrophy the extent of chronic inflammatory response has been suggested to be linked to the severity of dystropathology (Pescatori et al., 2007; Porter et al., 2002). Depletion of macrophages in the mdx mouse model at the early, acute peak of muscle pathology produced large reductions in lesions in the plasmalemma of muscle fibers (Wehling et al., 2001), showing that muscle macrophages that are present during the acute, degenerative stage of mdx dystrophy are highly cytolytic, and that they play a central role in the pathogenesis of muscular dystrophy.

Glucocorticoids, the most powerful anti-inflammatory and immunosuppressive agents available, are the only available treatment that allows to slow down disease progression in DMD patients (Horber and Haymond, 1990). However, it is well known from experience in chronic inflammatory diseases, not involving muscles, that long-term GC treatment causes muscle atrophy secondary to protein catabolism and muscle proteolysis (Horber and Haymond, 1990). Therefore, the efficacy of GC treatment in DMD patients is the net benefit of positive effects (suppression of inflammation) and negative effects (muscle catabolism). A better understanding of the inflammatory process in the dystrophic muscle and of the mediators involved might open alternative therapeutic perspectives.

In the present study we have focused our attention on the inflammatory cytokine interleukin 6 (IL6), based on the evidence that it is highly expressed in DMD patients and in mdx mouse model and it also plays a major role in inducing the transition from an acute neutrophilic infiltrate to a chronic type mononuclear cell infiltrate (Gabay, 2006).

IL6 is a pleiotropic cytokine that can contribute to the positive regulation of muscle homeostasis under physiological conditions and to the negative regulation of the muscle phenotype under some pathological circumstances (Fuster and Walsh, 2014; Munoz-Canoves et al., 2013). The complex actions of IL6 may be linked to the different manners by which this cytokine signals at the plasma membrane and by the different signaling pathways that can activate (Fuster and Walsh, 2014; Munoz-Canoves et al., 2013; Pedersen and Febbraio, 2008). Based on the activation of either classic or trans-signaling, IL6 can promote markedly different cellular responses. IL6 trans-signaling, which requires the soluble IL6R (sIL6R), is pro-inflammatory, whereas classic IL6 signaling, mediated by membrane-bound receptor, promotes regenerative or anti-inflammatory activities of the cytokine (Rose-John, 2012).

Circulating IL6 levels are normally very low or undetectable and are markedly increased in several diseases associated with inflammation, inducing the transition from an acute to a chronic inflammatory response (Gabay, 2006). IL6 is also locally and transiently produced in response to exercise and injury, and it plays an important role in satellite cell proliferation and muscle growth (Kurek et al., 1996; Serrano et al., 2008). In contrast, increased muscle proteolysis was found after administration of high doses or long-term exposure to IL6 in rodents (Goodman, 1994; Haddad et al., 2005; Tsujinaka et al., 1995). We recently demonstrated that treatment of C2C12 myogenic cells with recombinant IL6 resulted in a dramatic inhibition of myoblast differentiation (Pelosi et al., 2014). Interestingly, the inhibition of IL6 activity in an injured muscle, using an anti-interleukin-6 receptor antibody, promotes muscle regeneration via immune modulation (Fujita et al., 2014), whereas the inhibition of the IL6 intracellular mediator, namely the Jak/Stat pathway, stimulates muscle regeneration in both aged and dystrophic mice (Price et al., 2014; Tierney et al., 2014). The opposite effects exerted by IL6 can be justified considering the concept of hormesis, in which a low dose of a substance/molecule is stimulatory/homeostatic and a high dose is inhibitory/catabolic. Moreover, the positive effects of IL6 are normally associated with its transient production and short-term action. In contrast, persistent inflammatory conditions, including muscular dystrophy, are associated with long-lasting elevated systemic IL6 levels. Thus, muscle tissue might benefit from low and transient increases in IL6, whereas it is damaged by exposure to persistently high levels of this cytokine (Scott et al., 1996).

In the current study, we investigated whether specific blockade of IL6 activities may represent a potential therapeutic to treat human DMD. We revealed that blockade of IL6 activity in mdx mice, using a neutralizing antibody against the IL6 receptor (moAb-Il6r), confers robustness to dystrophic muscle, impedes the activation of a chronic inflammatory response, significantly reduces necrosis, activates the circuitry of muscle differentiation and maturation. This results in a functional homeostatic maintenance of dystrophic muscle.

## 2. Materials and Methods

### 2.1. Mice

Wild type and mdx mice were maintained according to the institutional guidelines of the animal facility of the unit of Histology and Medical Embryology. Mdx male mice (C57BL10) (purchased from Jackson Laboratories) were injected subcutaneously, starting at 15 days of age, with control IgG1 or with the neutralizing monoclonal antibody MR16-1 (kindly provided by Chugai Pharmaceutical Co., Ltd) (Okazaki et al., 2002) to the murine Il6 receptor at a dose of 100 μg/g of body weight and then twice a week with 20 μg/g (for a total of 5 doses) in PBS. Mice were sacrificed at day 30 of age. All animal experiments were approved by the ethics committee of Sapienza University of Rome-Unit of Histology and Medical Embryology and were performed in accordance with the current version of the Italian Law on the Protection of Animals.

### 2.2. Patients

All DMD patients had a molecular genetic detection of a mutation in the dystrophin gene predicting a marked dystrophin-deficiency. Sera from 29 non-treated DMD patients, 22 DMD patients treated with glucocorticoids and from 26 age-matched healthy controls were also collected. The human studies have been reviewed by the ethics committee of Bambino Gesù Children's Hospital and have therefore been performed in accordance with the ethical standards laid down in an appropriate version of the 1964 Declaration of Helsinki. All persons gave their informed consent prior to their inclusion in the study.

### 2.3. Treadmill Tests

The treadmill tests were performed using the LE8700 Treadmill Control (Panlab sl) (Cassano et al., 2008). Four week old mdx and moAb-Il6r-treated mice were acclimated to treadmill running (5 m/min for 5 min, after which the speed was increased 1 m/min every 2 min up to 9 m/min) before the test was performed. The mice (minimum 4 animals/strain) ran on the treadmill at an inclination of 0° at 5 m/min for 5 min, after which the speed was increased 0.6 m/min every 1 min. The test was stopped when the mouse remained on the shocker plate for more than 20 s without attempting to re-engage the treadmill, and the time to exhaustion was determined. The test was repeated four times (every two days).

### 2.4. Evans Blue Staining and Confocal Microscopy

Intraperitoneal injection of Evans blue dye (EBD) (100 μl of 1% EBD per 10 g of body mass) was performed on a minimum of 5 animals/strain (wild type, moAb-Il6r-treated mdx, and IgG1-treated control mdx mice). Fluorescent fibers were viewed under an inverted microscope (Axioskop 2 plus; Carl Zeiss Microimaging, Inc.), and images were processed using Axiovision 3.1 and analyzed using Scion Image 4.0.3.2. software. Confocal microscopy (Leica Laser Scanning TCS SP2) was used to analyze the total intensity of EBD fluorescence, which represents the full amount of fluorescence held within the entire z-axis of the series, and the mean amplitude of fluorescence in mdx and moAb-