HHS Public Access

Author manuscript

Life Sci. Author manuscript; available in PMC 2021 September 15.

Published in final edited form as:

Life Sci. 2020 September 15; 257: 118069. doi:10.1016/j.lfs.2020.118069.

Genetic disruption of the inflammasome adaptor *ASC* has minimal impact on the pathogenesis of Duchenne muscular dystrophy in *mdx* Mice

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Abstract

Aim: Up-regulation of inflammasome proteins was reported in dystrophin-deficient muscles. However, it remains to be determined whether inflammasome activation plays a role in the pathogenesis of Duchenne muscular dystrophy. This study was therefore set out to investigate whether genetic disruption of the inflammasome pathway impacts the disease progression in *mdx* mice.

Main methods: Mice deficient in both dystrophin and ASC (encoded by *Pycard* [PYD And CARD Domain Containing]) were generated. The impact of ASC deficiency on muscular dystrophy of *mdx* mice were assessed by measurements of serum cytokines, Western blot, real-time PCR and histopathological staining.

Key findings: The pro-inflammatory cytokines such as TNF-α, IL-6, KC/GRO and IL-10 were markedly increased in the sera of 8-week-old *mdx* mice compared to WT. Western blotting showed that P2X7, caspase-1, ASC and IL-18 were upregulated. Disruption of ASC and dystrophin expression in the mdx/ASC$^{-/-}$ mice was verified by Western blot analysis. Histopathological analysis did not find significant alterations in the muscular dystrophy phenotype in *mdx/ASC*$^{-/-}$ mice as compared to *mdx* mice.

Significance: Taken together, our results show that disruption of the central adaptor ASC of the inflammasome is insufficient to alleviate muscular dystrophy phenotype in *mdx* mice.

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Conflict of interest

None

Keywords

ASC; DMD; *mdx* mice; inflammasome; inflammation; muscle regeneration

1. Introduction

Duchenne muscular dystrophy (DMD) is an X-linked inherited neuromuscular disorder[1]. It is the most common type of muscular dystrophy, caused by genetic mutations in *DMD*, the gene encoding dystrophin protein[2]. Dystrophin is involved in forming a mechanical link between the cytoskeleton and the extracellular matrix, and thereby stabilizes the plasma membrane integrity through its interactions with other dystrophin-glycoprotein complex (DGC)[3, 4]. The dystrophin protein is highly expressed in skeletal and cardiac muscles.

Studies of DMD prevalence suggest an average age at diagnosis in United States is from 5 to 9 years of age, affecting approximately 1 per 5,000 boys[5]. DMD has typically resulted in severe muscle wasting and loss of ambulation in their early teens, subsequently lead to respiratory and cardiac dysfunction and death between 20 and 40 years of age[1]. Activation of innate inflammatory response has been well characterized in the pathology of muscular dystrophy due to the sarcolemmal instability after the disruption of DGC complex with the release of cytoplasmic contents and increased inflammatory immune cells infiltration including neutrophil and monocytes to the damage site and muscle tissue[6–8]. In severe muscle damage, the muscle tissue fails to repair where the regenerative capacity of myofibers is compromised. Excessive inflammation has been reported to be associated with the muscle tissue loss and fibrosis by increased production of proinflammatory cytokines[9]. Clinically, the overexpression of the proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α), transforming growth factor beta (TGF-β), interleukin-1 (IL-1), and interleukin-6 (IL-6) has been reported in the muscle biopsies and serum samples from DMD patients, indicating that the inflammation may play an important role in pathogenesis of DMD[10–13].

In recent years, increasing attention has been focused on the sterile inflammatory response that is activated by a large multimeric protein complex, known as inflammasome. Inflammasomes belong to the innate immune system and are composed of a sensor protein, an adaptor protein and a zymogen, procaspase-1. Several inflammasome complexes have been described including NLRP3 (nucleotide-binding domain leucine-rich repeat (NLR) and pyrin domain containing receptor 3), NLRP1, NLRC4, AIM2 (absent in melanoma 2). To date, the most described inflammasome is NLRP3 inflammasome, which consists of NLRP3, PYCARD (PYRIN-PAAD-DAPIN domain [PYD] and caspase recruitment domain [CARD] containing) adaptor, frequently referred to as apoptosis-associated speck-like protein (ASC), and pro-caspase-1[14, 15]. Inflammasome is activated in the response to tissue damage or infection, which in turn activates procaspase-1 into active caspase-1. The active caspase-1 proteolytically activates cytokines interleukin−1β (IL−1β) and interleukin−18 (IL−18) and eventually induces an inflammatory form of cell death called pyroptosis[15–17]. Several studies have suggested a role of NLRP3 inflammasome in the pathogenesis of pulmonary hypertension, type 2 diabetes, liver damage, obesity-associated glomerular injury

and muscular dystrophy[18–21]. Rawat *et al* (2010) has previously shown that the inflammasomes components and pro-inflammatory cytokine such as ASC, pro-caspase-1 and IL−1β were upregulated in both dystrophin-deficient human (DMD) and *mdx* mouse skeletal muscle, indicating dystrophin-deficient induced activation of inflammasomes pathway. However, the contribution of the inflammasomes in the pathophysiology of DMD has not been well defined. This study aims to explore the effect of ASC deficiency on the pathology of muscular dystrophy.

2. Materials and methods

2.1 Mice.

All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the Ohio State University. The *mdx* (C57BL/10ScSn-Dmd*mdx*/J) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). ASC deficient mice[22] (a gift from Dr. Sutterwala) and *mdx* mice were intercrossed to generate mutant and control mice. Genotyping of ASC and *mdx* mice was performed by tail DNA PCR as previously described[22, 23]. All mice were maintained at The Ohio State University Laboratory Animal Resources in accordance with animal use guidelines. All animal studies were authorized by the Animal Care, Use, and Review Committee of The Ohio State University.

2.2 Serum cytokine detection by ELISA.

The blood samples were collected from 8-week-old male WT, *mdx* and *mdx*/ASC$^{-/-}$ mice before sacrificed. The blood was allowed to clot for 15 min to 30 min and centrifuged at 8000 rpm for 15 min in room temperature. The supernatant was collected as serum and stored at −80 °C for the cytokine detection assay. Cytokine levels were detected using MSD (Meso Scale Discovery, Rockville, MD) 10 panels V-PLEX mouse ELISA kit, following the manufacturer's protocol. The signal was collected with MESO QUICKPLEX SQ 120 and the data were analyzed using MSD Workbench 4.0 software.

2.3 Western blotting.

Quadriceps muscles were harvested from 8-week-old male WT, *mdx* and *mdx*/ASC$^{-/-}$ mice. The tissues were homogenized with KINEMATICA Polytron™ PT 1300D Homogenizers (Bohemia, NY) and lysed with cold RIPA buffer (150 mM Sodium Chloride, 1% Triton X100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, 5 mM EDTA, pH 7.4) supplemented with protease inhibitors. The lysates were then centrifuged at 20,000 g for 20 min and supernatant was collected for Western blotting. The protein concentration of the supernatant was determined using the Lowry assay (Bio-Rad Laboratories, Hercules, CA, USA). Equal amount of extracted protein samples was separated by SDS-PAGE (BioRad, 4–20%) and transferred onto PVDF membranes (0.45 μm). The blots were blocked for non-specific binding with 5% milk in Tris-buffered saline containing 0.1 % Tween 20 (TBS, pH 7.4) for 1 hour at room temperature with gentle shaking. After rinsing in TBS-T, the blots were incubated at 4°C overnight with primary antibodies against ASC (AL177, AG-25B-0006-C100, Adipogen International, San Diego, CA), caspase-1 (sc-514, Santa Cruz Biotechnology, Dallas, TX), P2X7 (ab109246, Abcam, Cambridge, MA), IL-18

(5180R; Biovision, Milpitas, California), IL−1β (ab205924, Abcam) and dystrophin (E2660, Spring Bioscience, Pleasanton, CA). The blots were washed in TBS-T and incubated with respective secondary antibodies conjugated to horseradish peroxidise for 2 hours at room temperature. The blots were developed using ECL Western blotting substrate (Pierce Biotechnology, Rockford, IL) and images were captured using the ChemiDoc system (Bio-Rad Laboratories). The densitometric analysis was performed using Image Lab™ software, version 5.2.1 (Bio-Rad Laboratories). The respective protein expression levels were normalized to the housekeeping protein β-actin.

2.4 Immunofluorescence staining and histology.

Frozen muscle sections of 8 μm were prepared from quadriceps of 8-week-old male WT, *mdx* and *mdx*/ASC$^{-/-}$ mice. Immunofluorescence staining was performed as described previously[24]. In brief, the slides were fixed with 4% paraformaldehyde for 15 minutes at room temperature and washed with PBS before blocked with 5% BSA for 1 hour. The slides were incubated with primary antibodies against eMyHC (F1.652, Developmental Studies Hybridoma Bank, University of Iowa, IA) and laminin α2 (4H8/2, Alexis, San Diego, CA) at 4 °C overnight. After that, the slides were washed with PBS and incubated with secondary antibodies (Alexa Fluor 488 goat anti-rat IgG, Invitrogen, Carlsbad, CA or Alexa Fluor 594 goat anti-mouse IgG, Invitrogen) for 1h at room temperature. The slides were sealed with VECTASHIELD Antifade Mounting Medium with DAPI (vector laboratory, Burlingame, CA). Embryonic-MyHC-positive muscle fibers were counted and expressed as percentage of total number of positive fibers over total number of fibers in 10 fields. All images were taken under a Nikon Ti-E fluorescence microscope, magnification, X200 (Nikon, Melville, NY).

2.5 Histology.

For histological examinations, frozen muscle sections of 8 μm were prepared from each group and were fixed in 4% paraformaldehyde for 15 minutes at room temperature. The slides were then proceeded to the standard protocol of hematoxylin and eosin (H&E), and Masson-trichrome staining to show myofiber morphology and assess collagen content. All images were taken under a Nikon Ti-E fluorescence microscope, magnification, X200 (Nikon, Melville, NY).

2.6 Serum Creatine Kinase Levels.

Creatine kinase (CK) levels were analyzed using the Creatine Kinase-SL assay kit (Sekisui Diagnostics, Lexington, MA) according to manufacturers' instructions. Briefly, 6 μL of diluted serum sample was added accordingly to the 96-well UV microplate and following by 300 μL of CK reagent. The plate was incubated at 30°C for 5 minutes and read in Flexstation 3 microplate reader (Molecular Devices, Sunnyvale, CA) for 2 min at 30 sec intervals.

2.7 Central nucleation counting

Frozen muscle sections were stained with anti-laminin α2 antibody to delineate muscle fibers, and VECTASHIELD® Mounting Medium with DAPI was used for nuclear staining. All fibers, except those in direct contact with fascia, were analyzed for the location of their

nuclei. For each sample group, the number of fibers with centrally localized nuclei relative to the total number of fibers was recorded. For each individual mouse, about 2500–3500 fibers were counted.

2.8 Gene expression of IL-18 and IL-1β.

Total RNA was extracted using RNeasy Mini Kit (Qiagen, USA) according to manufacturer's instruction. Then 5μg RNA was reverse transcribed using RevertAid RT Reverse Transcription Kit (Thermo Scientific, USA). Quantitative PCR was carried out using the Radiant™ SYBR Green qPCR kits (Alkali Scientific, Pompano Beach, FL). The IL-18 forward and reverse primer sequences were 5′-AAAGTGCCAGTGAACCC-3′ and 5′-TTTGATGTAAGTTAGTGAGAGTGA-3′ respectively. The IL-1β forward and reverse primer sequences were 5′-CACAGCAGCACATCAACAAG-3′ and 5′-GTGCTCATGTCCTCATCCTG-3′ respectively. Each assay was run on a LightCycler® 480 System (Roche Diagnostics Corporation, IN, USA) in triplicate and the results are calculated using the relative standard curve method. All gene expressions were normalized against the respective housekeeping gene GAPDH.

2.9 Statistics.

The data were expressed as mean ± SEM and analyzed with GraphPad Prism 5.0 software (San Diego, USA). Statistical significance was determined using one-way ANOVA followed by Bonferroni post hoc-tests or unpaired 2-tailed Student's t test whenever appropriate. A P value of less than 0.05 is regarded as significant.

3. Results

3.1 Serum inflammatory cytokine profile in mdx mice

When cells die by necrosis they stimulate a robust acute inflammatory response *in vivo*[25]. Repeated muscle injury in the absence of dystrophin occurs, which persistently activates the immune system and causes muscle inflammation. We first examined the inflammatory profile in *mdx* mice by measuring inflammatory cytokines in the serum samples of 8-week-old *mdx* mice. The pro-inflammatory cytokines such as TNF-α, IL-6, KC/GRO and IL-10 were significantly increased in *mdx* mice compared to WT (Figure 1). The serum levels of IL-1β appeared to be increased in *mdx* mice, although the difference did not reach statistical significance.

3.2 Up-regulation of inflammasome proteins in mdx muscles

A number of cellular contents such as uric acid, HMGB1, SAP130, IL-1α, IL-33, DNA, S100 proteins, heat shock proteins and others, are released upon necrotic cell injury, which are believed to contribute to death-induced inflammation. The NLRP3 inflammasome, part of innate immune system, has been known to participate in the sterile inflammatory response, which may further exacerbate muscle pathology in muscular dystrophy. Next, our Western blotting results demonstrated a significant increase of P2X7 receptor, an ATP-gated ion channel in *mdx* mice relative to WT control. As expected, the biomarkers for activation of inflammasomes protein complex were found upregulated including caspase-1, ASC and

IL-18, in muscle lysates from *mdx* mice (Figure 2). Our data also confirmed the signature protein expression of dystrophin was absent in *mdx* mice (Figure 2A).

3.3 Effect of ASC deficiency on muscle pathology in mdx mice

To study if blockade of the NLRP3 inflammasome ameliorates muscular dystrophy pathology, we generated a double knockout (DKO) mouse strain with deficiency in both dystrophin and ASC, an inflammasome adaptor protein. The mice genotyped by tail DNA PCR were verified by Western blot analysis. Disruption of dystrophin and ASC expression was confirmed in the muscle lysates of the mdx/ASC DKO mice (Figure 3).

Repeated muscle degeneration and regeneration is a histopathological hallmark of muscular dystrophy, as indicated by the presence of muscle necrosis and central nucleated muscle fibers (CNF). The *quadriceps* and *gastrocnemius* muscles were assessed in H&E staining. As shown in the top panel of Figure 4A, both the *mdx* and *mdx*/ASC DKO muscles exhibited various size of muscle fibers, presence of internal nuclei, and increased fibrotic area. To quantify some of the pathological features, we stained the muscle sections with laminin α2 (to demarcate the muscle fibers) and 4',6-diamidino-2-phenylindole (DAPI, to label nuclei) (Figure 4A, bottom). Both *mdx* and *mdx*/ASC DKO muscles had significantly increased CNFs as compared to WT controls, but there was no significant difference between *mdx* and *mdx*/ASC DKO muscles (Figure 4B). Due to the presence of smaller, regenerating muscle fibers in dystrophic muscles, the average cross-sectional area (CSA) of muscle fibers were significantly decreased in both *mdx* and *mdx*/ASC DKO muscles (Figure 4C). Again, we observed no difference in CSA between *mdx* and *mdx*/ASC DKO muscles (Figure 4C). Moreover, the fiber size distribution was similarly altered in *mdx* and *mdx*/ASC DKO muscles as compared to WT controls (Figure 4D). Consistently, we observed that the serum creatine kinase (CK) levels were similarly increased in both *mdx* and *mdx*/ASC DKO mice (Figure 4E).

To further investigate if ASC disruption has any impact on muscle regeneration in *mdx* mice, embryonic myosin heavy chain (eMyHC), a marker of newly regenerated muscle fibers, was assessed. Both *mdx* and *mdx*/ASC DKO muscles exhibited increased number of eMyHC-positive fibers compared to WT (Figure 5A & B). However, no statistically significant changes were observed between *mdx*/ASC DKO and *mdx*.

4. Discussion

In this study, we found that the inflammasome proteins were up-regulated in the skeletal muscle of *mdx* mice, which also displayed an altered inflammation cytokine profile in the serum. However, genetic disruption of the inflammasome adaptor ASC in the *mdx* mice did not significantly improve the muscle pathology. Our results cast doubt on the feasibility of targeting inflammasome as a therapeutic strategy for DMD.

During sterile tissue damage, the immune system, particularly the inflammasome, has been implicated in many pathological conditions including DMD. Rawat *et al.* (2010) showed an increased expression of ASC and capspase-1[20] in muscle biopsies of DMD patients. In consistent with these previous observations, we also found that the expression of P2X7,

ASC, caspase-1 an IL-1β were significantly increased. These findings suggest a potential role of inflammasome activation in the pathogenesis of muscular dystrophy. Indeed, a previous study showed that genetic ablation of NLRP3 appeared to reduce the muscle pathology, inflammation and oxidative stress in *mdx* mice[26]. Ghrelin, a circulating hormone was found to suppress muscle inflammation and ameliorate disease phenotype in *mdx* mice via inhibition of NLRP3 inflammasome[27]. In addition, several other studies have shown that pharmacological blockade of P2X7 receptors reduced muscle inflammation and improve muscle pathology in *mdx* mice[28–30].

Given the promising previous observations mentioned above, it is surprising that genetic disruption of the inflammasome adaptor protein ASC had little impact on the muscle pathology in dystrophic mice as shown by our present study. As a central player of the NLRP3 inflammasome, ASC is a bipartite molecule that contains both an N-terminal PYD and a C-terminal CARD, allowing it to bridge the sensor NLRP3 and the effector pro-caspase-1[32]. The deletion or silencing of ASC has been reported to reduce hypoxia-induced pulmonary hypertension and high fat diet-induced podocyte injury[18, 21]. It is unclear what exactly underlies the discrepancy between our study and others, however, numerous studies have shown that NLRP3 may act via an inflammasome-independent mechanism, which may or may not require ASC. For example, hypoxia induced significant increase of NLRP3, independent of ASC, caspase-1 and IL-1β, which mediated mitochondrial regulation in renal injury[31]. NLRP3-deficient and ASC-deficient mice show difference in survival and early renal dysfunction following renal ischemic injury[32]. NLRP3 deficiency or inhibition via β-hydroxybutyrate precursor 1,3-butanediol protected the mice from nephrocalcinosis-related chronic kidney disease (CKD), but IL-1 inhibitor had no such protective effect, suggesting that NLRP3 has an IL-1-independent function in CKD[33]. During the NLRP3-mediated Epithelial-Mesenchymal Transition process induced by TNF-α or TGF-β1, the cleaved caspase-1 and ASC speck were not detected in mesenchymal-like colon cancer cells, indicating that NLRP3 functions in an inflammasome-independent way[34]. Moreover, NLRP3 was found to be required for resistance to pneumococcal pneumonia, whereas caspase-1 and caspase-11 were dispensable in S. pneumoniae-infected mice[35]. Thus, it is possible that the ASC-independent role of NLRP3 is involved in muscle pathology of *mdx* mice. Even more complicated, the sterile inflammatory responses are often only partially reduced and in some cases seem largely independent of the inflammasome components as demonstrated by the use of NLRP3-deficient animals[36]. Other cellular processes such as autophagy may also be involved in tissue inflammation[37]. Therefore it is possible that the mechanisms operating *in vivo* are more complicated and may involve redundant pathways. Disruption of one aspect of the immune system may be well compensated by the presence of the other redundant pathways.

5. Conclusions

This study shows that ASC knockout has minimal impact on muscle pathology of *mdx* mice. This finding suggests that targeting ASC may not be sufficient for alleviating muscle pathology in DMD. Further investigations are needed to better understand the inflammasome-dependent and independent functions of ASC and NLRP3 in muscular dystrophy.

Acknowledgements

This work was supported by US National Institutes of Health grants R01-HL116546 and R01-AR064241 to R.H.

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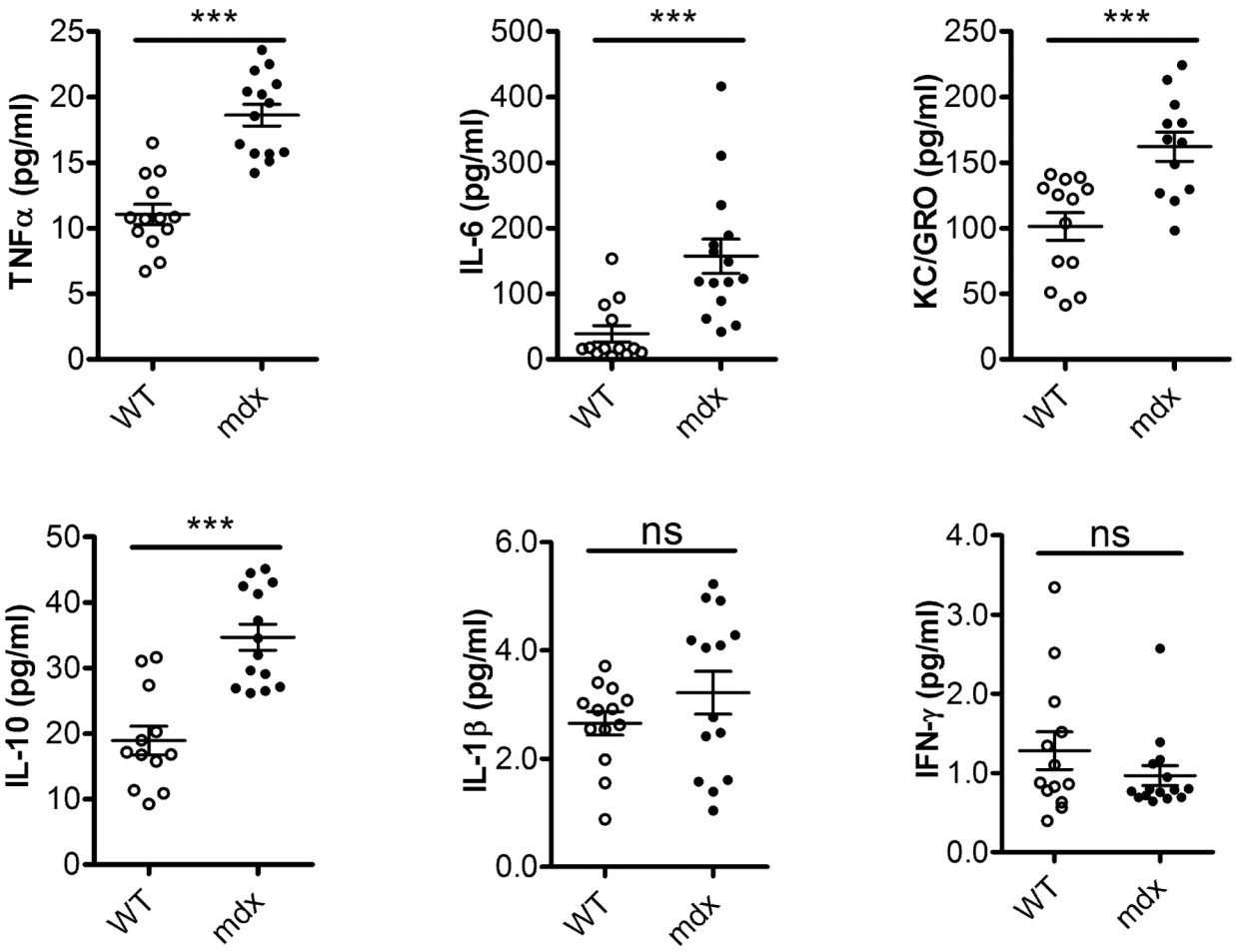

Figure 1.

Upregulation of pro-inflammatory cytokines in *mdx* mice. The serum cytokines profile was measured by ELISA in WT and *mdx* mice. The pro-inflammatory cytokines, TNF-α, IL-6, KC/GRO and IL-10 were increased in 8-week-old *mdx* mice compared to WT. Results are shown as mean ± SEM, n = 12–14, ***P < 0.001 compared with WT.

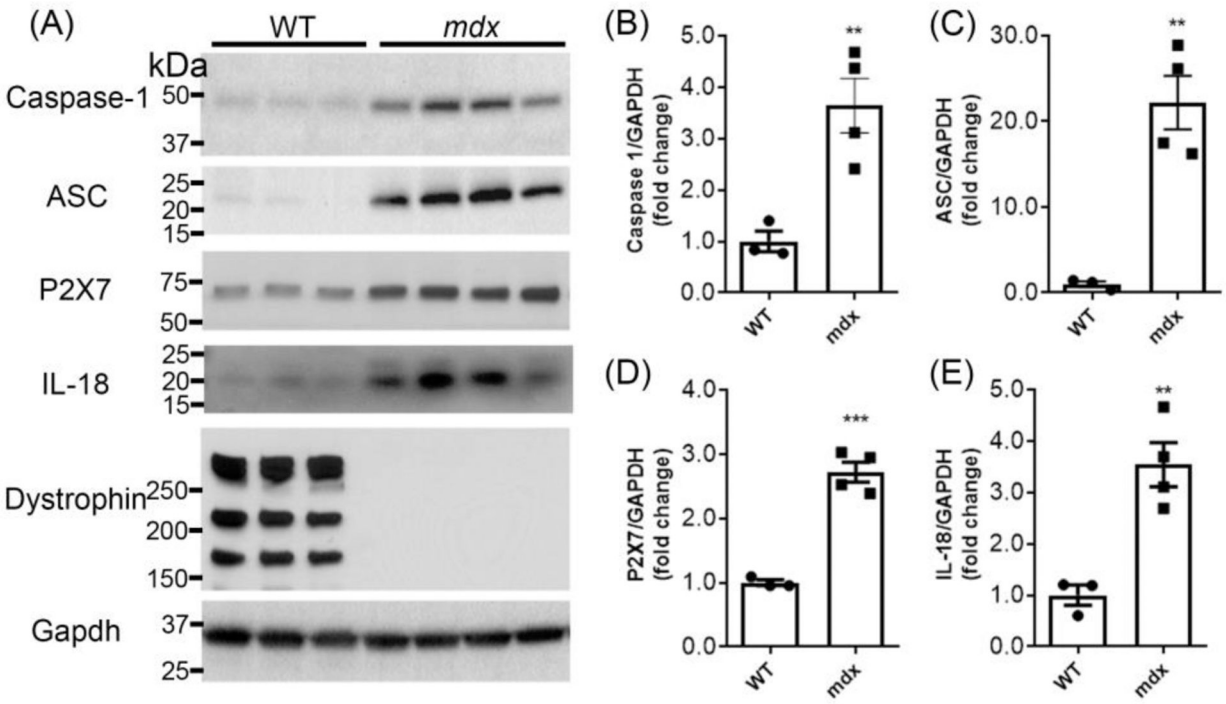

Figure 2.

Inflammasome signaling proteins upregulated in *mdx* mice. (A) The Western blotting shows upregulation of P2X7, caspase-1, ASC and IL-18 and absence of dystrophin protein in muscle lysates from *mdx* mice. (B-E) Graphs show quantitation analysis of Western blots. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with WT.

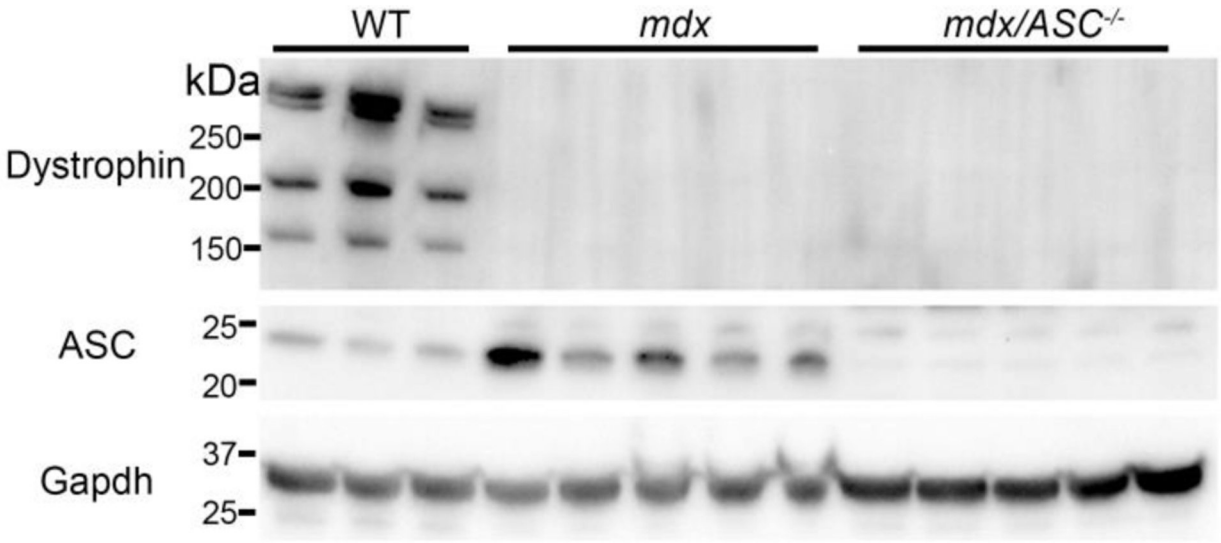

Figure 3.

Western blot analysis of muscle lysates from WT, *mdx* and *mdx*/ASC DKO mice. Western blotting shows upregulation of ASC and absence of dystrophin protein in muscle lysates from *mdx* mice and absence of both dystrophin and ASC protein in *mdx*/ASC DKO mice.

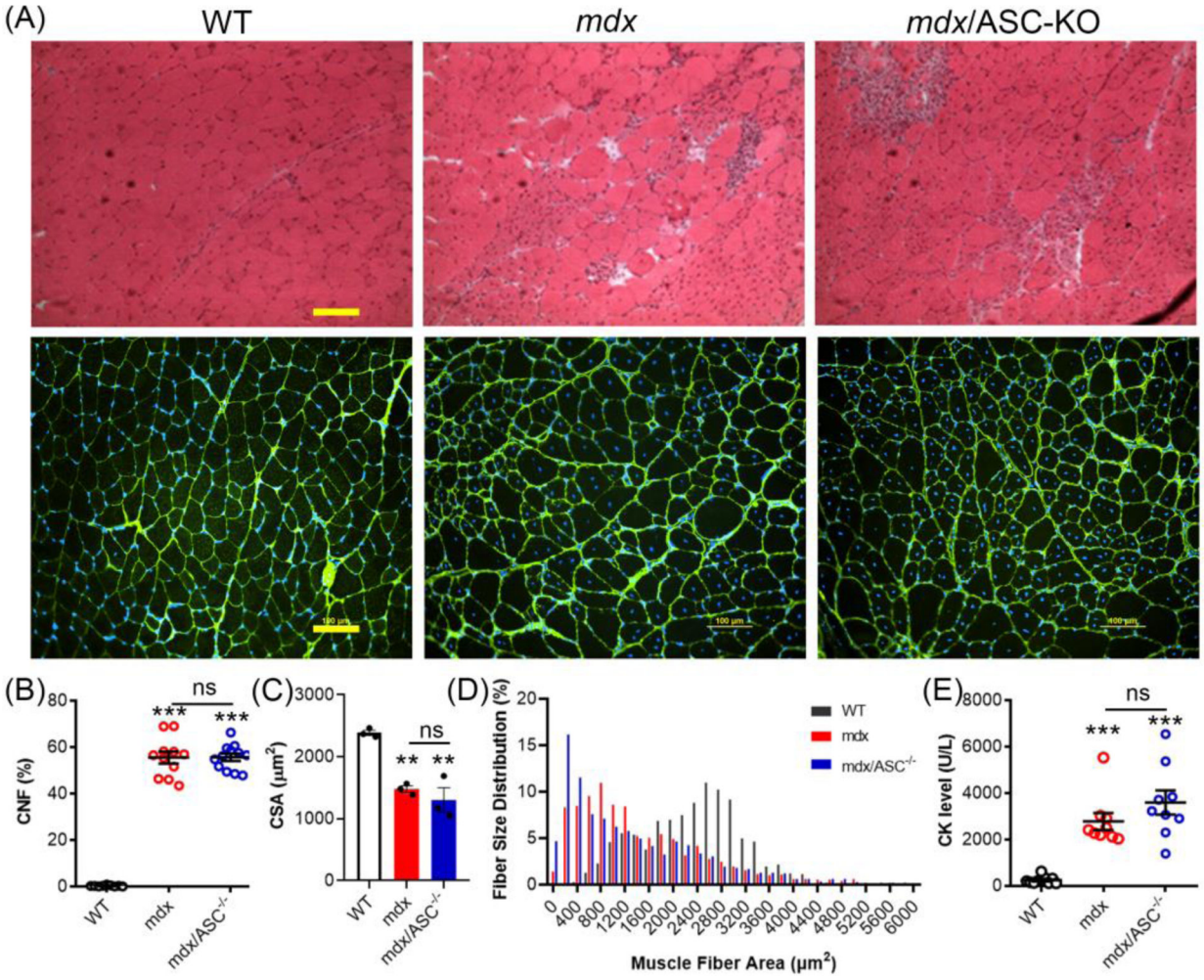

Figure 4.

ASC disruption had minimal impact on the dystrophic pathology of *mdx* mice. (A) H&E (top panel) and laminin α2/DAPI (bottom panel) stained histological sections of quadriceps from WT, *mdx* and *mdx*/ASC$^{-/-}$ mice. (B) Percentage of central nucleated fibers (CNF) in muscle sections of WT, *mdx* and *mdx*/ASC$^{-/-}$ mice. (C) Average cross-sectional area (CSA) of muscle fibers in WT, *mdx* and *mdx*/ASC$^{-/-}$ mice. (D) Muscle fiber size distribution of WT, *mdx* and *mdx*/ASC$^{-/-}$ mice. (E) Serum creatine kinase (CK) levels of WT, *mdx* and *mdx*/ASC$^{-/-}$ mice. ***$P < 0.001$ compared with WT; ns, not statistically significant.

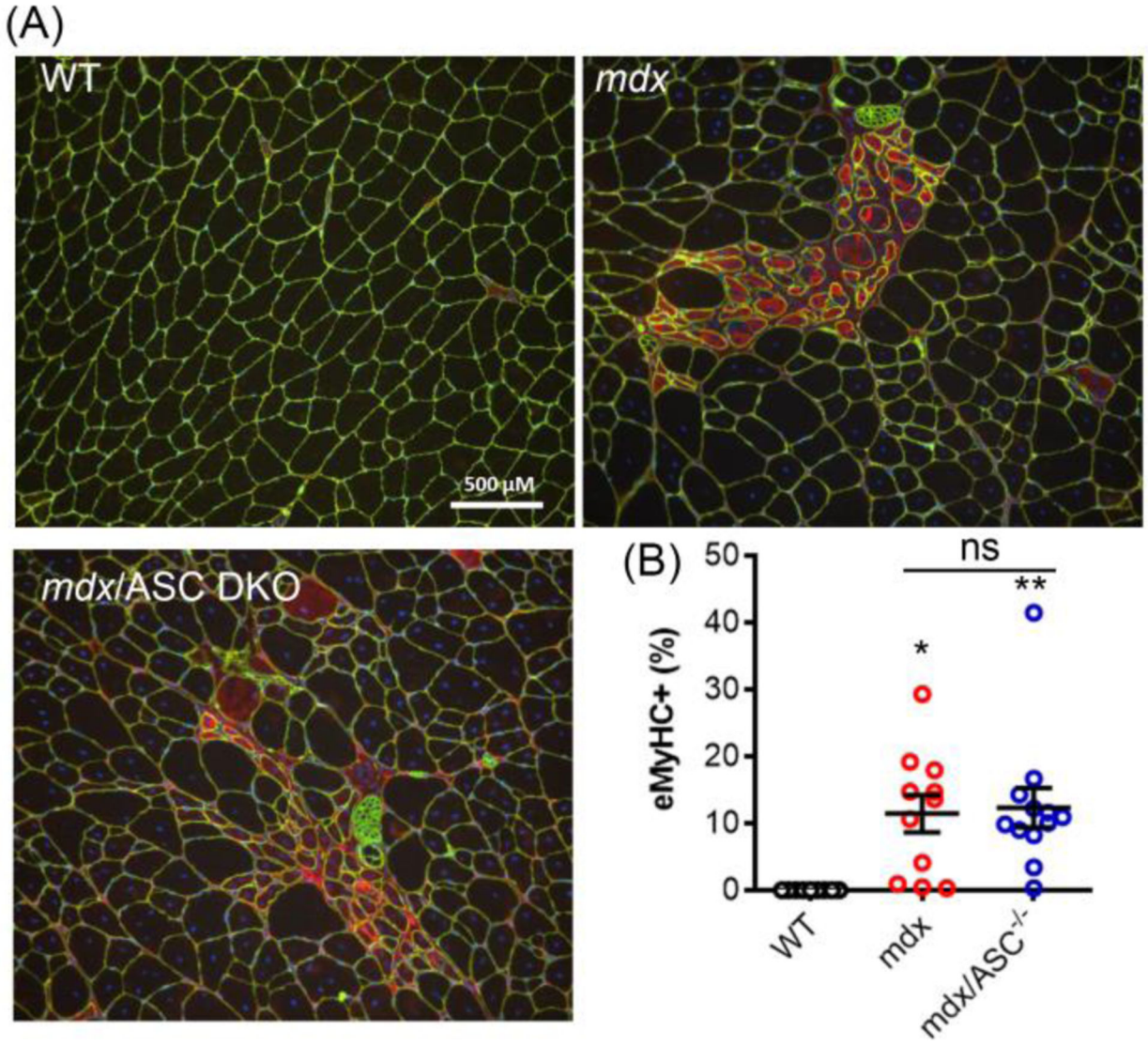

Figure 5.

Muscle regeneration was similar in *mdx* and *mdx*/ASC DKO mice. Representative immunofluorescence images (A) and quantitation (B) of eMyHC (red) from quadriceps of different mouse groups. Muscle fibers were co-stained with laminin 2α (green). *$P < 0.05$, **$P < 0.01$, compared with WT; ns, not statistically significant.