

Muscle Weakness in Myositis: MicroRNA-Mediated Dystrophin Reduction in a Myositis Mouse Model and Human Muscle Biopsies

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Objective. Muscle inflammation is a feature in myositis and Duchenne muscular dystrophy (DMD). Autoimmune mechanisms are thought to contribute to muscle weakness in patients with myositis. However, a lack of correlation between the extent of inflammatory cell infiltration and muscle weakness indicates that nonimmune pathologic mechanisms may play a role. The present study focused on 2 microRNA (miRNA) sets previously identified as being elevated in the muscle of patients with DMD—an “inflammatory” miRNA set that is dampened with glucocorticoids, and a “dystrophin-targeting” miRNA set that inhibits dystrophin translation—to test the hypothesis that these miRNAs are similarly dysregulated in the muscle of patients with myositis, and could contribute to muscle weakness and disease severity.

Methods. A major histocompatibility complex class I–transgenic mouse model of myositis was utilized to study gene and miRNA expression and histologic features in the muscle tissue, with the findings validated in human muscle biopsy tissue from 6 patients with myositis. Mice were classified as having mild or severe myositis based on transgene expression, body weight, histologic disease severity, and muscle strength/weakness.

Results. In mice with severe myositis, muscle tissue showed mononuclear cell infiltration along with elevated expression of type I interferon and NF-κB–regulated genes, including *Tlr7* (3.8-fold increase, $P < 0.05$). Furthermore, mice with severe myositis showed elevated expression of inflammatory miRNAs (miR-146a, miR-142-3p, miR-142-5p, miR-455-3p, and miR-455-5p; ~3–40-fold increase, $P < 0.05$) and dystrophin-targeting miRNAs (miR-146a, miR-146b, miR-31, and miR-223; ~3–38-fold increase, $P < 0.05$). Bioinformatics analyses of chromatin immunoprecipitation sequencing (ChIP-seq) data identified at least one NF-κB consensus element within the promoter/enhancer regions of these miRNAs. Western blotting and immunofluorescence analyses of the muscle tissue from mice with severe myositis demonstrated reduced levels of dystrophin. In addition, elevated levels of NF-κB–regulated genes, *TLR7*, and miRNAs along with reduced dystrophin levels were observed in muscle biopsy tissue from patients with histologically severe myositis.

Conclusion. These data demonstrate that an acquired dystrophin deficiency may occur through NF-κB–regulated miRNAs in myositis, thereby suggesting a unifying theme in which muscle injury, inflammation, and weakness are perpetuated both in myositis and in DMD.

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INTRODUCTION

The idiopathic inflammatory myopathies are a heterogeneous group of systemic connective tissue diseases characterized by chronic muscle inflammation and weakness. This group of diseases, which includes dermatomyositis (DM), polymyositis, and inclusion body myositis (IBM) (collectively referred to as myositis), affects both adults and children. Although rare, there is evidence that disease incidence is increasing (1). Histologic features of myositis include the following: 1) endomysial, perimysial, or perivascular infiltration by inflammatory cells, consisting predominantly of macrophages and T cells (2,3); 2) myofiber-specific expression of major histocompatibility complex (MHC) class I molecules; and 3) bouts of muscle degeneration/regeneration, fibrosis, and fat deposition (4). Of these, the most striking feature is the overexpression of MHC class I in the muscle of patients with myositis (5,6).

The role of the innate and adaptive immune systems in myositis is well known, but results recently reported suggest that nonimmune mechanisms also contribute to the disease (for review, see refs 7 and 8), as there is not a strong correlation between the extent of inflammatory cell infiltration and muscle weakness in patients with myositis (9). Interestingly, myositis muscle seems primed for inflammatory signaling events, since it inappropriately expresses Toll-like receptors (TLRs) (10,11) and MHC class I (5,6), which act as receptors that are normally confined to immune cells. Stimulation of TLR-7 and MHC class I activates the proinflammatory transcription factor NF- κ B. In addition, TLR-7 is a potent inducer of type I interferon (IFN) genes (12); this gene expression signature correlates with disease severity both in patients with DM (13–16) and in those with juvenile DM (15,17).

NF- κ B is a key regulator of inflammation in immune cells; however, it is also active in skeletal muscle under inflammatory conditions. Not surprisingly, in the muscle of patients with myositis, NF- κ B is highly activated and its activation corresponds to disease severity (18). Classic NF- κ B activation is triggered via inflammatory cytokines such as tumor necrosis factor (TNF), which is found at high levels in the muscle of patients with myositis (19). Nonclassic NF- κ B activation occurs through endoplasmic reticulum stress and autophagy; both of these processes/pathways are increased in myositis (20,21). It has been previously shown that muscle-specific MHC class I expression activates the NF- κ B pathway and turns on classic NF- κ B target genes such as *TNF* and *TLR7*, exacerbating inflammatory signaling (22). As further confirmation of its importance in disease pathogenesis, downstream gene targets of NF- κ B are highly up-regulated in myositis muscle (19,23).

At the molecular and histologic levels, there is significant overlap between inflammatory myopathies and other muscle disorders, in particular, Duchenne muscular dystrophy (DMD), Becker muscular dystrophy (BMD), and manifesting DMD female carriers (24,25). Similar to myositis, muscles of patients with DMD show increased TLR-7 expression (26), elevated NF- κ B activation (18,27), and overexpression of muscle-specific MHC class I (28). Previously, we described 2 distinct sets of microRNAs (miRNAs) that are elevated in DMD muscle. The first is an “inflammatory set” of miRNAs whose expression is increased in *mdx* mice and is reduced by administration of 2 different antiinflammatory drugs (29). The second set of miRNAs is a “dystrophin-targeting” set (termed dystrophin-targeting miRNAs [DTMs]). DTMs are elevated both in DMD and in BMD, and are prohibitory to dystrophin protein production in BMD and to exon-skipping-mediated dystrophin rescue in a mouse model of DMD (30). Some of these miRNAs show increased expression in inflammatory connective tissue disorders that overlap with myositis, including scleroderma, rheumatoid arthritis, and Sjögren’s syndrome, suggesting that inflammatory miRNAs and DTMs play a broader role in regulating chronic inflammation.

In mice, skeletal muscle-specific conditional overexpression of MHC class I induces myositis (3). Following MHC class I up-regulation, female mice develop early histologic and functional changes mirroring human myositis, while male mice develop a more mild disease (3). In the present study, we tested the hypothesis that inflammatory miRNAs and DTMs contribute to the pathologic processes of myositis by reducing dystrophin in muscle fibers.

MATERIALS AND METHODS

Mice. All mouse studies were performed with adherence to the NIH Guide for the Care and Use of Laboratory Animals. All experiments were conducted according to protocols within the guidelines of and with the approval of the Institutional Animal Care and Use Committee of Children’s National Medical Center. Animals were housed at room temperature in a 12-hour/12-hour light/dark cycle. Genotyping was carried out in all mice at age 3–4 weeks, as previously described (21). The mouse model of myositis used in this study is known as the HT mouse model of myositis, using (TRE)-H-2Kb (H) and mCK-tTA (T) mice, as has been described previously (3). For this study, transgene expression was induced by withdrawal of doxycycline from the drinking water of the mice at age 5 weeks, followed by analyses at ages 9–13 weeks. Female mice were used for all studies, as the disease manifestations are more severe in female mice.

BioPharma and Agada Biosciences. Dr. Fiorillo holds a patent for methods and agents to increase therapeutic dystrophin expression in muscle. No other disclosures relevant to this article were reported.

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Human muscle biopsy tissue. Muscle biopsy tissue samples from human subjects were obtained from a member of the Telethon Network of Genetic Biobanks (project no. GTB12001). The samples obtained included skeletal muscle, peripheral nerve samples, DNA samples, and cell lines. Further details can be found in the Supplementary Materials and Methods (available on the *Arthritis & Rheumatology* web site at <http://onlinelibrary.wiley.com/doi/10.1002/art.41215/abstract>).

Quantitative reverse transcription–polymerase chain reaction (qRT-PCR) analyses of miRNAs and messenger RNAs (mRNAs). Analyses of miRNA and mRNA expression by qRT-PCR were performed in muscle tissue samples in a manner as previously reported (29,30). Details can be found in the Supplementary Materials and Methods (<http://onlinelibrary.wiley.com/doi/10.1002/art.41215/abstract>).

Western blot analysis. Proteins from muscle were collected using a lysis buffer containing 75 mM Tris HCl (pH 6.8), 10% sodium dodecyl sulfate, 10 mM EDTA, and 5% 2-mercaptoethanol. Protein samples (25 µg) were separated using a 3–8% Tris–acetate gel (Bio-Rad) and then transferred onto a nitrocellulose membrane (20V at 4°C) overnight, followed by a 1-hour pulse (80V at 4°C). Antibodies used included the DYS-1 mouse monoclonal antibody against dystrophin (1:100; Novacastra), and a rabbit polyclonal antibody against vinculin (1:1,000, clone N3C1; GenTex). Horse-radish peroxidase–conjugated anti-mouse immunoglobulin or anti-rabbit goat immunoglobulin (1:5,000; Millipore) were used as secondary antibodies. Enzyme chemiluminescence (Pierce) was used for detection of the proteins. The blots were scanned digitally with a ChemiDoc Touch Imager.

Hematoxylin and eosin (H&E) staining and histologic grading of the muscle tissue sections. Sections of muscle tissue (8 µM) were cut from frozen mouse quadriceps muscles or from frozen human muscle biopsy tissue. Sections were air-dried for 1 hour on SuperFrost Plus slides and then stained with H&E. Stained slides were imaged on a VS120 Virtual Slide microscope (Olympus) at 20× magnification. Details on the grading of histopathologic features of the muscle can be found in the Supplementary Materials and Methods (<http://onlinelibrary.wiley.com/doi/10.1002/art.41215/abstract>).

Immunofluorescence. Frozen muscle sections (8 µM) were dried for 1 hour on SuperFrost Plus slides and then stored at –80°C. For staining, slides were thawed for 1 hour at room temperature and then fixed in either ice-cold acetone (4°C) for 10 minutes or 4% paraformaldehyde for 10 minutes at room temperature. Further details on the primary and secondary antibody dilutions and incubation times can be found in the Supplementary Materials and Methods (<http://onlinelibrary.wiley.com/doi/10.1002/art.41215/abstract>).

Evaluation of force contractions on isolated skeletal muscle. Extensor digitorum longus (EDL) muscle was isolated from live anesthetized mice prior to their euthanization, and contractile properties of the skeletal muscle were then measured *ex vivo*. Details can be found in the Supplementary Materials and Methods (<http://onlinelibrary.wiley.com/doi/10.1002/art.41215/abstract>).

Bioinformatics analysis. We examined each miRNA gene promoter by bioinformatics analysis, to gain insight into the mechanisms of response to treatment, as has been previously reported (29). Details on the identification of NF-κB/RelA binding sites can be found in the Supplementary Materials and Methods (<http://onlinelibrary.wiley.com/doi/10.1002/art.41215/abstract>).

RESULTS

Variable disease severity in mice with myositis. In the present study, we utilized female mice that overexpress MHC class I, and we performed our studies after withdrawal of doxycycline from the drinking water of the mice at age 5 weeks. At 4–8 weeks after doxycycline withdrawal, we observed variable disease severity among the mice, allowing us to investigate the molecular factors driving disease progression.

We initially observed that mice with a lower body weight at the time of euthanization appeared to have a more aggressive disease. To determine whether the body weight of mice is related to transgene expression, we plotted the weight of the mice at the time of euthanization against the extent of transgenic MHC class I expression in the most severely affected muscle (quadriceps). As shown in Figure 1A, body weight and transgene expression showed a strong inverse correlation (Spearman's rho = –0.9146), thus corroborating our observations that mice with a lower body weight develop a more severe disease, which can likely be attributed to increased MHC class I expression.

The scatter plot of the correlation between body weight and transgene expression revealed 2 distinct mouse phenotypes: HT mice with mild myositis (HT-M) and HT mice with severe myositis (HT-S). Mice classified in the HT-S myositis group weighed 12–16 grams at the time of euthanization, and their expression of muscle-specific MHC class I was at a level of >300-fold relative to wild-type (WT) mice. Mice classified in the HT-M myositis group weighed 17–20 grams and showed MHC class I transgene expression at a level of ≤300-fold relative to WT mice.

To determine whether differences in transgene expression were reflected in the muscle pathology, we used a grading system on H&E-stained muscle tissue from the HT-M and HT-S groups of mice. The muscle of mice with severe myositis showed 1) higher endomysial inflammation with invasion into the myofibers, 2) more highly variable muscle fiber size, and 3) increased necrosis and degeneration, with a mean histologic grade of 2.3 (on a scale of 0–3) ($P < 0.0001$ versus WT). In contrast, the muscle of mice with mild myositis showed 1) less endomysial

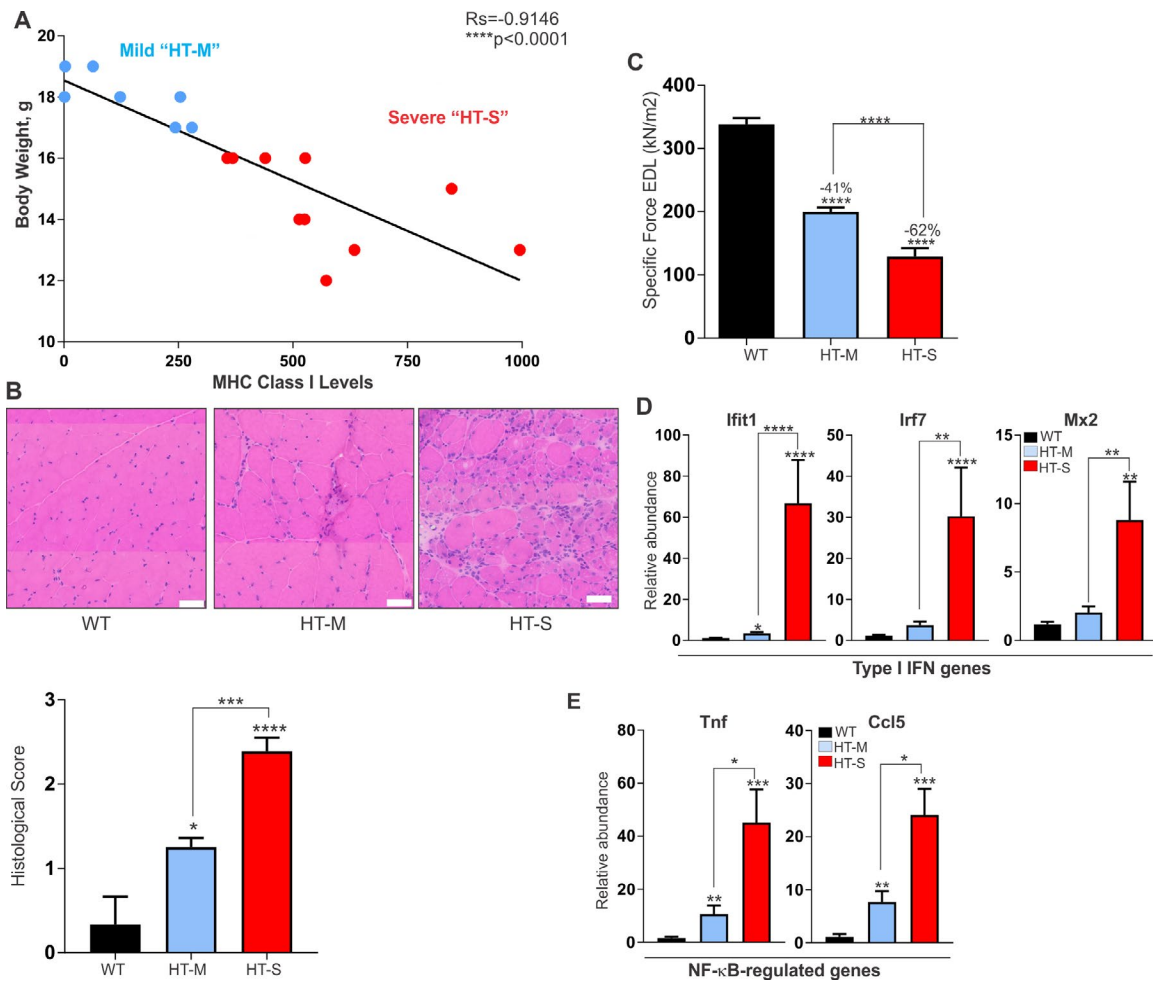


Figure 1. Variable disease severity in the HT mouse model of myositis. **A**, Classification system used for grouping HT mice according to the severity of myositis, based on body weight plotted against the extent of major histocompatibility complex (MHC) class I transgene expression. HT-M = mice with mild myositis, body weight of >16 grams, and MHC class I expression of ≤ 300 -fold relative to wild-type (WT) mice. HT-S = mice with severe myositis, body weight of ≤ 16 grams, and MHC class I expression of >300-fold relative to WT mice. Correlations were determined using Spearman's correlation coefficients. **B**, Representative images of histologic staining with hematoxylin and eosin (top) and histologic scores of staining (bottom) of the quadriceps muscle from WT mice and mice in the mild or severe myositis groups ($n = 5$ WT, $n = 7$ HT-M, and $n = 10$ HT-S). Bars = 50 μ M. **C**, Isolated measurements of specific force contractions of the extensor digitorum longus (EDL) muscle in each group of mice ($n \geq 10$ per group). **D** and **E**, Type I interferon (IFN) gene expression levels (genes for IFN-induced protein with tetratricopeptide repeats 1 [*Ifit1*], IFN regulatory factor 7 [*Irf7*], and MX dynamin like GTPase 2 [*Mx2*]) (**D**) and NF- κ B-induced gene expression (genes for tumor necrosis factor [*Tnf*] and C-C motif chemokine ligand 5 gene [*Ccl5*]) (**E**) in the quadriceps muscle of WT, HT-M, and HT-S mice ($n = 5$ WT, $n = 7$ HT-M, and $n = 10$ HT-S). Data were normalized to the values for 18S ribosomal RNA. Results in **B–E** are the mean \pm SEM. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$; **** = $P < 0.0001$ for the indicated comparisons or versus WT mice, by analysis of variance.

inflammation with no invasion into fibers, 2) less fiber size variation, and 3) little-to-no necrosis and degeneration, with a mean histologic grade of 1.3 ($P < 0.001$ versus HT-S) (Figure 1B).

To determine whether the differences in molecular and histologic features between the HT-S and HT-M groups of mice resulted in functional differences, we assessed the muscle strength of isolated EDL muscles ex vivo. Specific force in the EDL muscle was reduced by 62% in mice with severe myositis and was reduced by 41% in mice with mild myositis (each $P < 0.0001$ versus WT mice) (Figure 1C). Collectively, these data show that mice classified as HT-S indeed display a more severe disease as compared to mice classified as HT-M.

Type I IFN signature and NF- κ B activation in mice with severe myositis.

Results of recent studies have suggested that molecules induced by type I IFNs play a mechanistic role in the pathogenesis of myositis (13–16); this is most commonly observed in adult and juvenile patients with DM (15, 17). To determine whether type I IFN gene expression was related to disease severity in our study, we performed qRT-PCR on 3 type I IFN signature genes, the genes for IFN-induced protein with tetratricopeptide repeats 1 (*Ifit1*), IFN regulatory factor 7 (*Irf7*), and MX dynamin like GTPase 2 (*Mx2*). Muscle tissue from mice with severe myositis showed significant increases (~10–80-fold) in the relative expression of all 3 genes as compared to WT mice ($P < 0.0001$ for *Ifit1*

and *Irf7*, and $P < 0.01$ for *Mx2*) (Figure 1D). In comparison, muscle tissue from mice with mild myositis showed modest increases in *Irf1*, *Irf7*, and *Mx2*, with only the levels of *Irf1* reaching a statistically significant difference compared to WT mice ($P < 0.05$) (Figure 1D).

We have previously shown that NF- κ B is highly activated in human inflammatory myopathies and mouse models of myositis (22). To determine whether NF- κ B activation is associated with disease severity, we assessed the NF- κ B-activated genes *Tnf* and C-C motif chemokine ligand 5 gene (*Ccl5*). Both *Tnf* and *Ccl5* followed a similar pattern as that of type I IFN signature genes. In the muscle of mice in the HT-S group, the levels of both *Tnf* and *Ccl5* were highly increased over the levels in WT mice ($P < 0.001$) (Figure 1E). In the HT-M group of mice, NF- κ B-induced gene expression in the muscle also showed significant elevations compared to WT mice ($P < 0.01$), but to a lesser extent.

In response to their cognate ligands, TLRs induce both type I IFN and inflammatory (NF- κ B) gene expression

(31). Investigators have recently observed myofiber-specific expression of TLR-7 in several muscle disorders (10,26,32). Because we observed elevated expression of both types of gene expression signatures in mice with severe myositis, we next assessed TLR-7 expression in both the HT-M and HT-S myositis groups of mice. Using immunofluorescence, we observed an almost complete absence of TLR-7 from WT mouse muscle (Figure 2A). In contrast, the muscle of mice with mild myositis showed modest TLR-7 staining, while the muscle of mice with severe myositis showed strong TLR-7 staining (Figure 2A).

Using confocal microscopy, we observed colocalization of TLR-7 and lysosome-associated membrane protein 1 (LAMP-1), a marker of late endosomes/early lysosomes, in the mouse muscle (Figure 2B; positive controls are shown in Supplementary Figure 1, available on the *Arthritis & Rheumatology* web site at <http://onlinelibrary.wiley.com/doi/10.1002/art.41215/abstract>), indicating

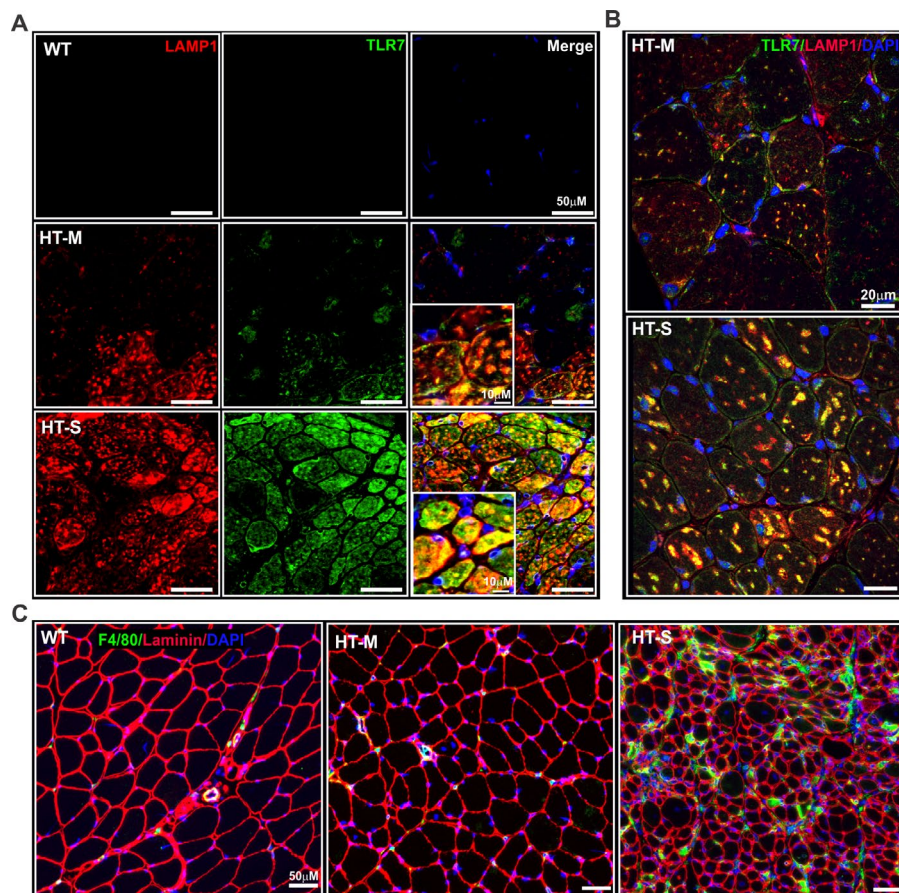


Figure 2. Increased Toll-like receptor 7 (TLR-7) staining and macrophage infiltration in the muscle of mice with severe myositis. **A** and **B**, Representative images of the quadriceps muscle of a WT mouse, mouse with mild myositis, and mouse with severe myositis, immunolabeled with an antibody against TLR-7 (green) and lysosome-associated membrane protein 1 (LAMP-1) (red). DAPI counterstaining was used to visualize nuclei (blue). Images in **A** were obtained with a VS-120 scanning microscope at 20 \times magnification, and images in **B** are from a second set of immunostained muscle tissue sections visualized using confocal microscopy. In both **A** and **B**, colocalization of TLR-7 and LAMP-1, a marker of late endosomes/early lysosomes, is an indication that TLR-7 is localized to the endosomes and is in an activated state. **C**, Muscle tissue from HT-M and HT-S mouse quadriceps stained with an antibody against F4/80, which recognizes a glycoprotein expressed by murine macrophages (green), and with an antibody against laminin (red), which confirms the integrity of the muscle tissue, with DAPI counterstaining of the nuclei (blue). See Figure 1 for other definitions.

that TLR-7 was in an activated state (33). Analyses by qRT-PCR corroborated the immunofluorescence data, showing elevated levels of *Tlr7* in the muscle of mice with severe myositis (see Supplementary Figure 2A, available on the *Arthritis & Rheumatology* web site at <http://onlinelibrary.wiley.com/doi/10.1002/art.41215/abstract>). In addition, qRT-PCR analyses indicated that *Lamp1* gene expression was increased in the muscle of mice with severe myositis; however, the difference compared to WT mice did not reach significance (see Supplementary Figure 2B [<http://onlinelibrary.wiley.com/doi/10.1002/art.41215/abstract>]).

Signaling through TLR-7 promotes macrophage recruitment and invasion into muscle fibers. Immunofluorescence analyses of mouse macrophage marker F4/80 showed increased macrophage infiltration in the muscle of mice with severe myositis

as compared to WT mice or mice with mild myositis (Figure 2C). This observation was confirmed by qRT-PCR analyses of a macrophage-specific marker, endothelial growth factor-like module-containing mucin-like hormone receptor-like 1 (*Emr1*). Analyses by qRT-PCR showed that the muscle of mice with severe myositis expressed elevated levels of *Emr1* ($P < 0.05$) (see Supplementary Figure 2C [<http://onlinelibrary.wiley.com/doi/10.1002/art.41215/abstract>]). Taken together, these data suggest that progression of myositis involves distinct changes in the molecular signature of myofibers, which begin to take on an innate immune-like state via expression of type I IFN signature genes, NF- κ B-regulated genes, and myofiber-specific expression of TLR-7, all of which may result in increased macrophage recruitment.

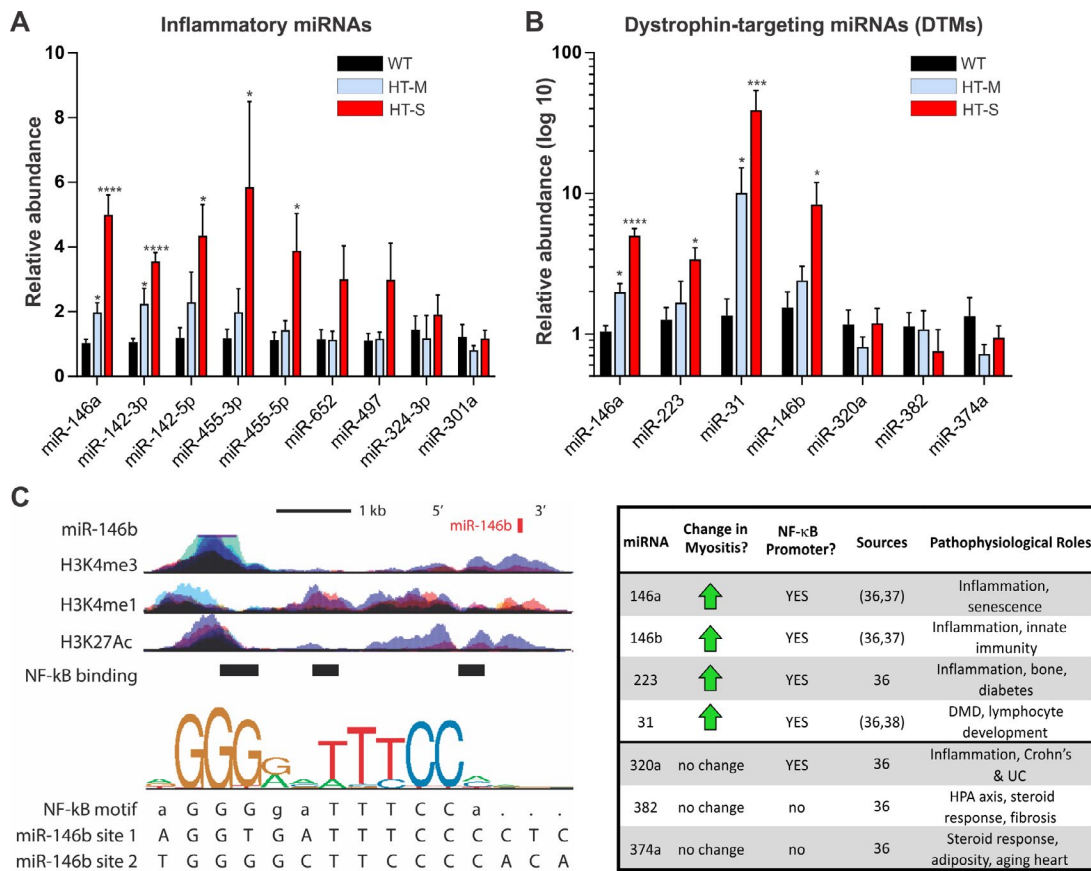


Figure 3. Expression of inflammatory microRNAs (miRNAs) and dystrophin-targeting miRNAs (DTMs) in the quadriceps muscle of mice classified as having severe or mild myositis disease. **A** and **B**, The relative abundance of inflammatory miRNAs (**A**) and DTMs (**B**) was analyzed by quantitative reverse transcription–polymerase chain reaction in the quadriceps muscle from each group of mice. Data were normalized to the values for *sno202*. Results are the mean \pm SEM ($n = 5$ WT, $n = 6$ HT-M, and $n = 8$ HT-S). * = $P < 0.05$; *** = $P < 0.001$; **** = $P < 0.0001$ versus WT mice, by analysis of variance. **C**, Transcription factor (NF- κ B) binding sites and histone (H3) modifications that mark regulatory regions were examined using chromatin immunoprecipitation sequencing data from ENCODE. Binding motifs for each transcription factor were identified through the Factorbook repository. Left Top, Schematic diagram of the gene locus for miR-146b, illustrating the binding sites of 3 neighboring DNA loci bound directly by NF- κ B. Epigenetic modification maps show the location of histone modifications associated with active promoters (H3K4me3) and poised/active enhancers (H3K4me1/H3K27Ac). Left Bottom, Sequence logo pictogram of the base frequency at NF- κ B binding sites with the consensus NF- κ B motif. Two representative NF- κ B binding site sequences near miR-146b are also shown. Right, Summary of promoter analysis and literature data for all DTMs, indicating each miRNA and known factors associated with its transcriptional regulation. DMD = Duchenne muscular dystrophy; UC = ulcerative colitis; HPA = hypothalamic–pituitary–adrenal axis (see Figure 1 for other definitions).

Association between muscle levels of inflammatory and dystrophin-targeting miRNAs with the severity of myositis. To further characterize the molecular changes that drive myositis disease progression, we analyzed 2 defined sets of miRNAs in the mouse quadriceps muscle. The first set encompassed 9 miRNAs (termed inflammatory miRNAs) that we previously found to be elevated in dystrophic mice. This

set of miRNAs are regulated by NF- κ B and their expression decreases in response to antiinflammatory treatment (29). Of these, the levels of 5 miRNAs (miR-146a, miR-142-3p, miR-142-5p, miR-455-3p, and miR-455-5p) were significantly elevated in the muscle of mice with severe myositis compared to WT mice (~4–6-fold increase, $P < 0.05$) (Figure 3A). In the muscle of mice with mild myositis, the levels of miR-142-3p

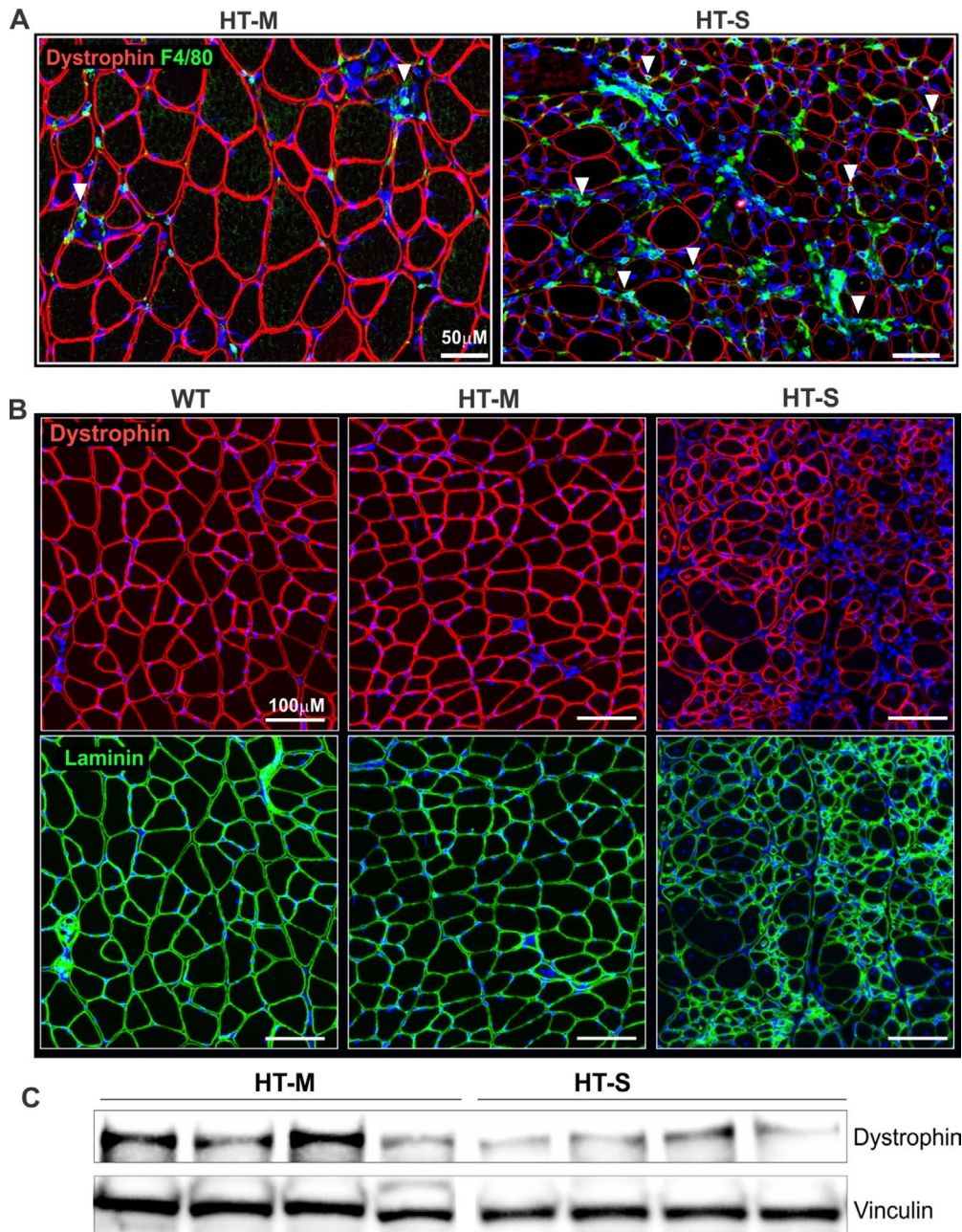


Figure 4. Reduced dystrophin levels in mice with severe myositis. **A**, Representative images of the quadriceps muscle of mice in the mild or severe myositis groups, immunolabeled with an antibody against dystrophin (red) and macrophage staining (F4/80; green). **Arrowheads** indicate regions with high macrophage infiltration (F4/80; green) where neighboring myofibers are observed to show diminished dystrophin levels (red). **B**, Representative images of the quadriceps muscle of mice in the mild or severe myositis groups compared to WT mice, using immunolabeling with an antibody against dystrophin (red). An anti-laminin antibody (green) was used as a control to show sarcolemmal integrity of the muscle tissue. DAPI counterstaining was used to visualize nuclei (blue). **C**, Western blotting for dystrophin, using protein extracts from the tibialis anterior muscle of mice in the mild or severe myositis groups ($n = 4$ per group). Vinculin was used as a loading control and was run on the same Western blot. See Figure 1 for definitions.

and miR-146a were also significantly elevated compared to WT mice, but to a lesser extent.

We next assayed a second defined set of miRNAs (termed DTMs), which we previously reported to be up-regulated both in patients with DMD and in those with BMD. These miRNAs bind to the dystrophin 3'-untranslated region (3'-UTR) to inhibit its translation. Analyses by qRT-PCR showed that the expression levels of 4 of 7 DTMs (miR-146a, miR-223, miR-31, and miR-146b) were highly elevated (~3–40-fold increase, $P < 0.05$) in the quadriceps muscle of mice with severe myositis compared to WT mice (Figure 3B). We also observed significantly elevated levels of miR-146a and miR-31 in the muscle of mice with mild myositis compared to WT mice, but to a lesser extent.

Because we observed elevated TLR-7 expression in myositis muscles, we also assessed expression of 3 miRNAs known to activate TLR-7: miR-21 (34), miR-29a (34), and let-7 (35). In our analyses, miR-21 was undetectable in all samples assessed, while the expression of miR-29a and let-7 showed no significant differences between WT, HT-M, and HT-S mouse muscles (see Supplementary Figure 3, available on the *Arthritis & Rheumatology* web site at <http://onlinelibrary.wiley.com/doi/10.1002/art.41215/abstract>).

DTMs (miR-146a and miR-223) are induced by TNF in dystrophic myotubes, suggesting that they are regulated by NF- κ B (30). To gain insight into DTM regulation in myositis, we performed bioinformatics analyses on chromatin immunoprecipitation sequencing (ChIP-seq) data at the sites of each miRNA promoter/enhancer region. Bioinformatics analyses showed that all 4 elevated DTMs possessed sites that are directly bound by NF- κ B at consensus elements within their promoter/enhancer regions (Figure 3C) (36). These elements overlapped with corresponding ChIP-seq data indicating the presence of histone modifications, all of which corresponded to active regions of transcriptional regulation (H3K4me3, H3K27Ac, and H3K4me1). These findings are supported by previous studies in which miR-146a (37), miR-146b (37), miR-223 (38), and miR-31 (39) were found to be regulated by NF- κ B. Taken together, these data suggest that both defined sets of miRNAs play a key role in the progression of myositis, and that NF- κ B regulates their expression.

Reduction in dystrophin expression linked to the severity of myositis. Our laboratory has demonstrated that DTMs inhibit the translation of dystrophin, and that DTM levels inversely correlate with the levels of dystrophin in the muscle of patients with BMD and in the exon-skipping-treated muscle of *mdx* mice in the model of DMD (30). To determine whether elevated levels of DTMs reduce dystrophin in myositis muscle, we performed dystrophin immunostaining in the quadriceps muscle of WT mice and mice in the HT-M and HT-S groups. Dystrophin expression was markedly reduced in the muscle of mice with severe myositis as compared to mice with mild myositis, most notably in areas of inflammation as shown by macrophage (F4/80)

staining (Figure 4A; arrowheads indicating macrophage staining alongside myofibers showing reduced dystrophin).

We next performed immunostaining for both dystrophin and laminin to better visualize the muscle fibers lacking dystrophin. In the HT-S group of mice, muscle fibers that lacked dystrophin stained positively for laminin, indicating the presence of intact muscle fibers (Figure 4B). To corroborate the immunofluorescence findings, we also performed Western blotting on muscle tissue extracts from the HT-M and HT-S groups of mice. The results similarly showed that dystrophin levels were reduced in muscle from mice with severe myositis (Figure 4C).

To determine the relevance of these findings to human disease, we obtained human muscle biopsy tissue from patients with DM and those with IBM as well as muscle biopsy tissue from healthy control subjects. Analysis by H&E staining revealed different levels of histologic severity in the muscle tissue based on the extent of inflammation and muscle fiber size variability. According to the histologic findings, we partitioned the patients into "histologically mild" or "histologically severe" cohorts. Two biopsy samples from patients with DM and 1 from a patient with IBM were grouped as "histologically mild," and the remaining 3 biopsy samples (from patients with IBM) were grouped as "histologically severe." Representative images are shown in Figure 5A.

We next assessed MHC class I (HLA-B) gene expression in human muscle biopsy tissue. Those in the histologically severe group showed a ~6-fold elevation in MHC class I expression ($P < 0.05$), whereas muscle tissue from the histologically mild group showed no significant elevation in MHC class I expression above the levels in healthy controls. Muscle with histologically severe disease showed elevated levels of NF- κ B-driven gene expression, with evidence of increased expression of *TNF* and *CCL5* (each $P < 0.05$ versus healthy controls), whereas muscle with histologically mild disease showed no appreciable up-regulation in gene expression (Figure 5B). We also observed elevated expression of *TLR7* in histologically severe muscle ($P < 0.05$ versus healthy controls) (Figure 5B), consistent with our observations in the muscle of mice with severe myositis.

We assessed type I IFN gene expression and found no significant differences between the healthy control and histologically mild or histologically severe groups of human muscle biopsy tissue. We did, however, observe elevated expression of type I IFN genes in 1 of 2 muscle biopsy samples from patients with DM (data not shown), consistent with previous studies in which it was shown that this gene expression signature is a hallmark of DM, particularly in muscles that have perifascicular atrophy (15,17).

We next assessed levels of NF- κ B-regulated DTMs, which were found to be elevated in the human muscle tissue with histologically severe disease. We observed that the expression of both miR-146a ($P < 0.10$) and miR-146b ($P < 0.001$) was increased in histologically severe muscle biopsy tissue compared to healthy controls, while that from the histologically mild group

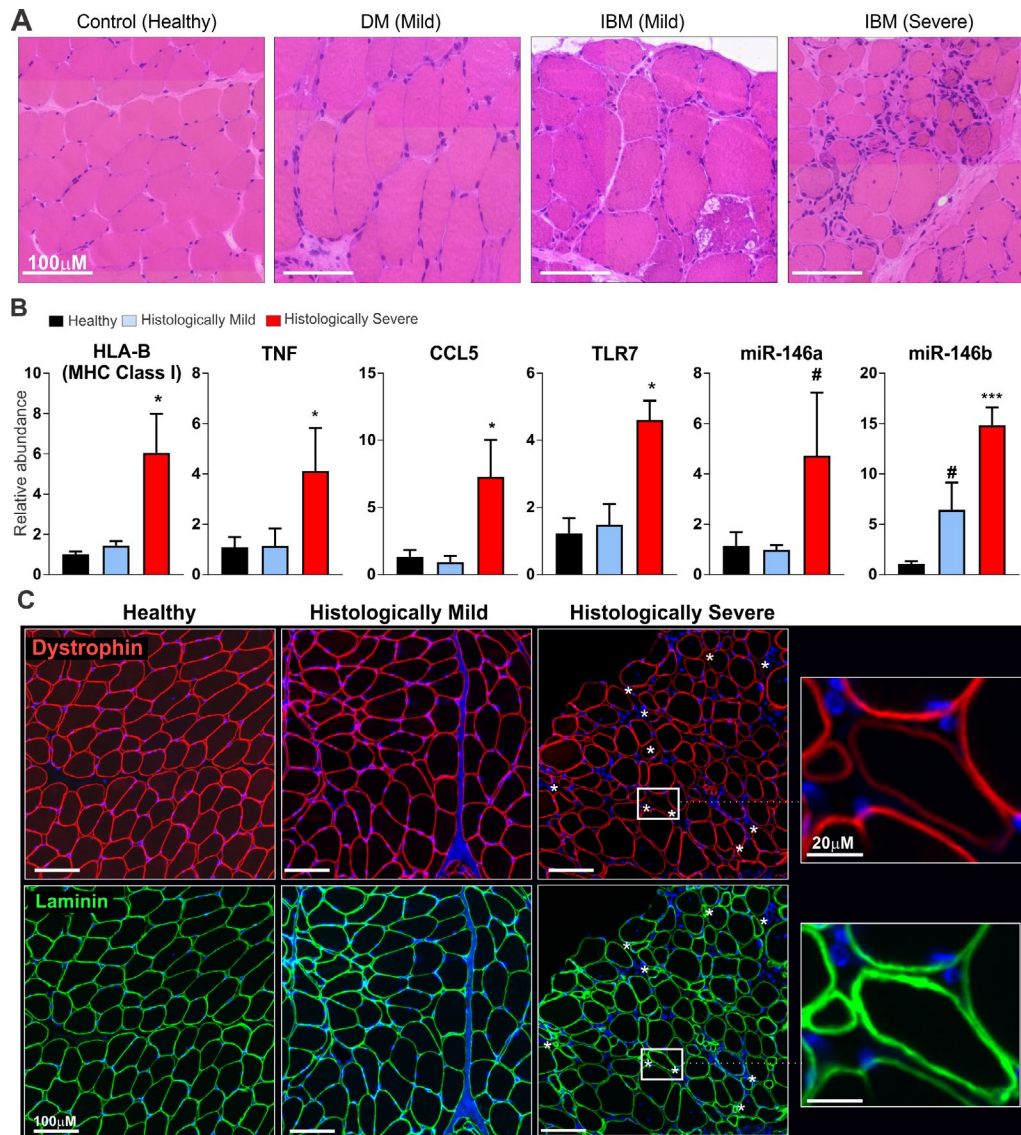


Figure 5. Elevations in major histocompatibility complex (MHC) class I expression, NF- κ B-driven gene expression, and dystrophin-targeting microRNAs (miRNAs) and reduction in dystrophin levels in histologically severe human muscle biopsy tissue. **A**, Representative images of hematoxylin and eosin–stained human muscle biopsy tissue obtained from a healthy control, a patient with dermatomyositis (DM) classified as having histologically mild muscle disease, and 2 patients with inclusion body myositis (IBM), of whom 1 was classified as having histologically mild muscle disease and 1 as having histologically severe muscle disease. **B**, Gene and miRNA expression levels in human muscle biopsy tissue from each group ($n = 3$ per group). Data for mRNAs were normalized to the values for *Hprt*, and data for miRNAs were normalized to the values for *RNU48*. # = $P < 0.10$; * = $P < 0.05$; *** = $P < 0.001$ versus healthy controls, by Student's *t*-test. **C**, Representative images of human muscle biopsy tissue from a healthy control, a patient with histologically mild muscle disease, and a patient with histologically severe muscle disease, using immunolabeling with an antibody against dystrophin (red). An anti-laminin antibody (green) was used as a control to show sarcolemmal integrity of the muscle tissue. **Asterisks** in the histologically severe muscle highlight muscle fibers in which laminin staining is uniform, but dystrophin staining is either reduced, discontinuous, or absent. DAPI counterstaining was used to visualize nuclei (blue). TNF = human gene for tumor necrosis factor; TLR7 = human gene for Toll-like receptor 7.

showed no differences in miR-146a expression, but did show slightly higher miR-146b levels in comparison to healthy muscles ($P < 0.10$) (Figure 5B).

We then performed immunofluorescence analysis of dystrophin expression in the human muscle biopsy samples, using sarcolemmal-localized laminin as a control for the integrity of the muscle fibers. Whereas staining for dystrophin

in the healthy control and histologically mild muscle tissue was uniform, we found areas of reduced and discontinuous dystrophin staining in histologically severe muscle tissue (Figure 5C).

Collectively, our data suggest a mechanism whereby increased TLR-7 signaling activates inflammation and NF- κ B-driven miRNAs to promote muscle weakness through reduced

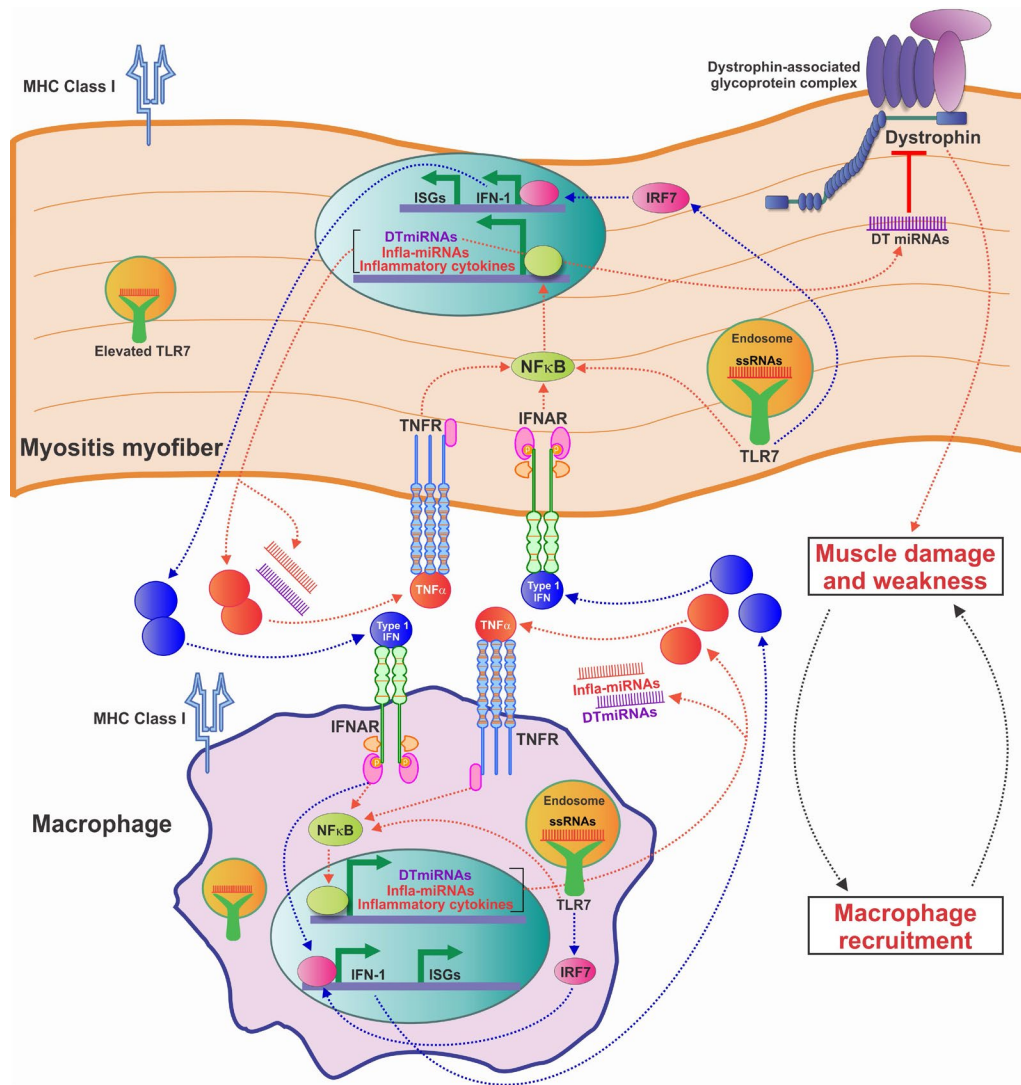


Figure 6. Model of the hypothesized self-amplifying feedback loop between macrophages and myofibers in myositis. Progression of myositis is driven by myofibers that adopt a more immune-like state, as evidenced by inappropriate expression of Toll-like receptor 7 (TLR-7) and major histocompatibility complex (MHC) class I. TLR-7 activates NF-κB–driven gene expression, eliciting the release of inflammatory cytokines and promoting macrophage recruitment. Activation of NF-κB–driven genes such as tumor necrosis factor (TNF) and interferon-α/β (IFN) genes leads to a feed-forward loop in which these cytokines activate their receptors (TNFR and IFNAR, respectively), thereby enhancing NF-κB activation. TLR-7 additionally activates the IFN regulatory factor 7 gene (*IRF7*), driving production of IFN-1, which in turn activates IFNAR and expression of type I IFN–stimulated genes (ISGs), which includes *IRF7*. NF-κB also triggers expression of inflammatory and dystrophin-targeting microRNAs (miRNAs) (infla-miRNA and DT-miRNA, respectively), leading to reduction in the levels of dystrophin and muscle weakness, which in turn causes increased release of proinflammatory signals. All of these components contribute to a positive, self-amplifying inflammatory feedback loop. ssRNAs = single-stranded RNAs.

expression of dystrophin (refer to model in Figure 6). Furthermore, elevated levels of DTMs may be responsible for the reduced and discontinuous dystrophin expression observed in myositis muscle, both in the HT mouse model and in patients with idiopathic inflammatory myopathies.

DISCUSSION

Herein, we utilized a mouse model of myositis using HT mice with variable disease severity to better understand the molecular

and cellular pathways that contribute to myositis. Previous reports have described the common occurrence of a chronic disease progression in these mice, in which reduced locomotor activity is seen as early as 1 month after doxycycline withdrawal, and clinical and histopathologic signs are observable at ~2 months, which thereafter become profound and severe by 4–6 months (3). Due to the sensitivity of our assay, the analyses performed herein in mice at 1–2 months after being taken off doxycycline enabled us to identify early molecular aberrations and additionally to measure weakness in isolated EDL mouse muscles ex vivo.

The cohort of HT mice in our study possessed variable rates of disease progression. Thus, because of the progressive nature of the model, we predict that the disease in HT mice with mild myositis will become more severe through time.

We classified mice into 2 distinct cohorts: HT-M mice with low transgenic expression of muscle-specific MHC class I, and HT-S mice with high MHC class I transgene expression. Comparing these 2 groups and age-matched WT littermates, we found that mice in the HT-S group developed a more severe disease, as shown by a lower body weight, histologic severity of the disease in the muscle tissue, and reduced muscle strength/weakness, as well as elevated levels of type I IFN and NF- κ B gene signatures. Furthermore, mice in the HT-S group showed inappropriate expression of endolysosomal-localized TLR-7 within myofibers and increased macrophage infiltration as visualized by F4/80 staining. Correspondingly, the muscle of mice in the HT-S group displayed markedly increased levels of miRNAs from 2 defined sets: an inflammatory set previously described in a DMD mouse model (29), and a dystrophin-targeting set previously described both in patients with BMD and in exon-skipping-treated muscle of *mdx* mice that model DMD (30). All of the miRNAs that showed elevated expression in severe myositis muscle from these sets possessed at least one NF- κ B regulatory element within their promoter or enhancer regions.

Using both immunofluorescence and Western blot analyses, we demonstrated that dystrophin was reduced in the muscle of mice with severe myositis. To corroborate our data in the HT mouse model, we additionally analyzed human muscle biopsy samples from patients with myositis, and found that histologically severe muscles had elevated levels of MHC class I, elevated NF- κ B-driven gene expression, and high levels of miR-146a and miR-146b. Human muscle with histologically severe myositis also showed patchy/reduced levels of dystrophin immunostaining. Importantly, the muscle biopsy samples classified as severe were from patients with IBM, whereas the muscle biopsy samples from patients with DM were classified as mild. This is consistent with previous studies in which MHC expression was shown to be more prominent in the muscle of patients with IBM (40). Collectively, our data suggest a model whereby progression of myositis is driven by myofibers adopting a more immune-like state, as evidenced by the inappropriate expression of TLR-7 and MHC class I. TLR-7 activates NF- κ B-driven gene expression, eliciting release of inflammatory cytokines and leading to macrophage recruitment. NF- κ B-driven gene expression also triggers an NF- κ B feed-forward loop, in which production of inflammatory cytokines, including TNF, further turns on NF- κ B. TLR-7 activation also leads to production of type I IFN, which, in turn, triggers expression of NF- κ B and type I IFN-stimulated genes. NF- κ B additionally activates dystrophin-targeting miRNAs, which down-regulate dystrophin protein levels. Taken together, these findings indicate that this combination of events appears to contribute to a self-amplifying inflammatory feedback loop of muscle dam-

age and inflammation. As dystrophin is required for maintenance of mechanical stability in the muscle, our observations suggest that reduced dystrophin levels in myositis muscle may contribute to muscle weakness (Figure 6).

Many signaling pathways described herein are similarly over-expressed or overactive in DMD. For instance, in muscles from patients with DMD there is strong activation of multiple components of the innate immune system before the onset of clinical symptoms, including elevated signaling from TLRs (TLR-4 and TLR-7), NF- κ B activation, and expression of MHC class I molecules. It is recognized that there is substantial overlap between the histopathologic presentation of myositis and muscular dystrophies, specifically DMD, BMD, and manifesting DMD female carriers. Transcriptional profiling also shows shared pathways between these diseases (41). In a few case studies, DMD female carriers were found to be initially misdiagnosed as having myositis, further illustrating the multitude of confounding factors that blur the boundaries between the 2 diseases (25,42). In the present study, we describe miRNAs whose levels were elevated in both diseases. These similarities give rationale for the development of therapeutic strategies that could target shared pathways in DMD and myositis.

Recent studies have demonstrated inappropriate expression of TLR-7 in myofibers from myositis patients (10,11), and have shown that this increase in expression exacerbates disease (43). Similarly, TLR-7 levels are elevated in the myofibers of patients with DMD at an early age (<1 year of age) (27). Because of the high TLR-7 levels observed in DMD muscle, it is postulated that myofiber-derived RNA molecules may be the most potent of all of the contributing damage-associated molecular patterns (DAMPs), as they serve as the natural ligand for TLR-7 (44). In support of this, we have previously found that a more common feature of DMD and BMD muscle is miRNA up-regulation as opposed to miRNA down-regulation (30). Further, recent studies have suggested that the overabundance of specific miRNAs may trigger activation of TLR-7 (34,35). Collectively, high levels of miRNAs in muscle disease and the misexpression of TLR-7 in myofibers seem to be alluding to a mechanism whereby diseased muscle itself is primed to activate a strong inflammatory response.

TLR-7 is trafficked to the endolysosomal compartment when activated (45). In the present study we observed elevated levels of endosomally localized TLR-7 in muscle affected with severe myositis. We also observed elevated LAMP-1 expression, which may be indicative of impaired autophagy (21,46). It has been reported that autophagy is required for the activation of NF- κ B (47), and that TLR-7 and its ligands regulate autophagy (48,49). Thus, these seemingly intertwined pathways make attractive targets for myositis and perhaps for other inflammation-associated muscle disorders.

MicroRNAs are responsible for fine-tuning gene expression in all cell and tissue types, including muscle and immune cells. When the balance of miRNAs is shifted in a specific tissue type,

gene expression programs are altered, driving tissue from a healthy state to a diseased state. In HT mice with severe myositis, we found significant up-regulation of 5 of the 9 miRNAs from a predefined “inflammatory” set (miR-146a, miR-142-3p, miR-142-5p, miR-455-3p, and miR-455-5p) and 4 of the 7 miRNAs from a predefined “dystrophin-targeting” set (miR-146a, miR-223, miR-31, and miR-146b). We additionally found elevated levels of miR-146a and miR-146b in human muscle biopsy tissue with a classification of histologically severe disease. All of these miRNAs are regulated by the inflammatory transcription factor NF- κ B (36), and some, in turn, regulate key components of the NF- κ B signaling pathway. This highlights the importance of NF- κ B signaling in the pathogenesis of myositis. We will discuss the significance of these miRNAs below.

Levels of miR-146a are highly up-regulated in mice with severe myositis. Moreover, miR-146a is induced by NF- κ B in immune cells (37) and is expressed directly in muscle (30). In diseases in which chronic inflammation is present, miR-146a levels are highly elevated both in the serum and in the tissue affected by disease (50–57). Similarly, miR-146b is overexpressed in severe myositis, is induced by TNF/NF- κ B (37,58), and is elevated in the serum of patients with inflammatory disorders such as inflammatory bowel disease (57). Both miR-146a and miR-146b are defined as DTMs, and we have shown that they down-regulate dystrophin by binding to its 3'-UTR (30). The miR-142 family is highly expressed in monocytes (59) and lymphocytes (60). We and others have reported increased miR-142 levels in DMD as well as in other genetic muscular dystrophies (29,61). The miR-142 locus possesses 13 NF- κ B binding sites within its promoter/enhancer region, illustrating the interdependence of its expression and inflammatory signaling. Increased expression of miR-31 has been reported in DMD patients, BMD patients, and *mdx* mice (30,62–64). In addition, miR-31 regulates dystrophin expression by targeting its 3'-UTR (30,65), and its expression is induced by NF- κ B (39). Collectively, the miRNAs described herein seem to be uniquely primed to shift the balance toward a proinflammatory phenotype as they become increasingly expressed in diseased muscle.

A few early studies noted reduced and discontinuous dystrophin staining in human muscle biopsy tissue from patients with myositis (66,67). Herein, we investigated dystrophin levels in a mouse model of myositis and in human muscle biopsy tissue after we observed elevated levels of DTMs in HT mice with severe myositis. Immunofluorescence analysis revealed that the quadriceps muscle of mice affected with severe myositis possessed a reduced and mosaic pattern of dystrophin staining that partly resembled the pattern that has been previously observed in the muscles of patients with BMD or the muscles from DMD female carriers. Reduced dystrophin expression was associated, in part, with the extent of inflammation, as shown by macrophage (F4/80) staining. Given that DTMs are regulated by inflammation, it is plausible that DTMs within the muscle microenvironment could dictate the local expression of dystrophin. As disease progresses, both inflammation and dystrophin reduction could “spread” through the muscle, as patches of damaged mus-

cle communicate through DAMPs to healthy areas, in turn activating inflammatory signaling, further reducing dystrophin levels, and causing muscle weakness. Interestingly, reduced levels of dystrophin have been reported in other muscle disorders in which dysregulated NF- κ B signaling and chronic inflammation are present (67–69). Loss of dystrophin could provide a possible mechanism for muscle weakness in myositis which, to date, is not fully understood. It will be particularly important to study dystrophin deficiency in other muscle disorders, especially those characterized by high inflammation, to determine whether a secondary dystrophin deficiency could contribute to disease.

In this study, we have provided novel insights into the mechanisms driving muscle weakness and disease progression in myositis, using a murine model and human muscle biopsy tissue. Future work will be aimed at determining 1) whether the microRNAs described herein also play a role in other muscle/inflammatory disorders, and 2) whether these miRNAs could serve as therapeutic targets in muscle disease.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Drs. Nagaraju and Fiorillo had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Kinder, Heier, Tully, Fiorillo.

Acquisition of data. Kinder, Heier, Tully, Van der Muelen, Fiorillo.

Analysis and interpretation of data. Kinder, Heier, Tully, Hoffman, Nagaraju, Fiorillo.

ADDITIONAL DISCLOSURE

Author Hoffman is the Treasurer, Secretary, and Vice President of Research of ReveraGen BioPharma as well as the President and CEO of AGADA Biosciences. Author Nagaraju is the President and CEO of ReveraGen BioPharma as well as the Founder and Vice President of AGADA Biosciences.

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