ANIMAL MODELS

Dysferlinopathy Promotes an Intramuscle Expansion of Macrophages with a Cyto-Destructive Phenotype

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Dysferlinopathies are a group of muscular dystrophies resulting from a genetic deficiency in *Dysf*. Macrophages, highly plastic cells that mediate tissue repair and destruction, are prominent within dystrophic skeletal muscles of dysferlinopathy patients. We hypothesized that *Dysf*-deficient muscle promotes recruitment, proliferation, and skewing of macrophages toward a cyto-destructive phenotype in dysferlinopathy. To track macrophage dynamics in dysferlinopathy, we adoptively transferred enhanced green fluorescent protein–labeled monocytes into *Dysf*-deficient BLA/J mice with age-related (2 to 10 months) muscle disease and *Dysf*-intact (C57BL/6 [B6]) mice. We detected an age- and disease-related increase in monocyte recruitment into *Dysf*-deficient muscles. Moreover, macrophages recruited into muscle proliferated locally and were skewed toward a cyto-destructive phenotype. By comparing *Dysf*-deficient and -intact monocytes, our data showed that *Dysf* in muscle, but not in macrophages, mediate intramuscle macrophage recruitment and proliferation. To further elucidate macrophage mechanisms related to dysferlinopathy, we investigated *in vitro* macrophage-myogenic cell interactions and found that *Dysf*-deficient muscle i) promotes macrophage proliferation, ii) skews macrophages toward a cyto-destructive phenotype, and iii) is more vulnerable to macrophage-mediated apoptosis. Taken together, our data suggest that the loss of *Dysf* expression in muscle, not macrophages, promotes the intramuscle expansion of cyto-destructive macrophages likely to contribute to dysferlinopathy. Identifying pathways within the *Dysf*-deficient muscle milieu that regulate cyto-destructive macrophages will potentially uncover therapeutic strategies for dysferlinopathies. (*Am J Pathol* 2017, 187: 1245–1257; <http://dx.doi.org/10.1016/j.ajpath.2017.02.011>)

Dysferlinopathies are a group of autosomal recessive neuromuscular diseases caused by a genetic deficiency in dysferlin (*Dysf*), a protein involved in muscle repair.^{1,2} The absence of *Dysf* triggers progressive skeletal muscle wasting that often begins suddenly in a patient's late teens or early adulthood, and within a decade the patient has limited use of his or her limbs.¹ Macrophage-rich unrelenting inflammation in muscles from patients and mice is characteristic of this form of muscular dystrophy.³ The intramuscle accumulation of macrophages is notably more abundant in dysferlinopathy than in other inflammatory muscle diseases.⁴ Despite the prominence of macrophages in dysferlinopathy, the role of these myeloid cells remains a mystery. Because there is no effective treatment for dysferlinopathy,

clarifying the mechanisms responsible for intramuscle macrophage expansion and the impact of macrophages in this illness will undoubtedly uncover novel therapeutic targets.

The burning question is whether macrophages in *Dysf*-deficient hosts are programmed to repair or destroy muscle. To probe for the role of intramuscle macrophages in nonresolving inflammation leading to muscular dystrophy, it is essential to understand normal muscle repair. Fundamentally, macrophage-mediated inflammation is a necessary

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antecedent to healing because it sets the stage for repair. Within this context, it is important to appreciate that macrophages are highly plastic myeloid cells with extremely diverse functions. For simplicity, macrophages are broadly divided into two subsets, classically activated (M1) cyto-destructive and alternatively activated (M2) tissue-reparative leukocytes.⁵ Within this framework macrophages are further subdivided according to polarization, gene expression, and function.⁶ With this stratification in mind, injured muscle initially recruits nondividing, proinflammatory monocytes to the damaged site.⁷ Once within the inflamed muscle, macrophages shift from a cyto-destructive M1 to a tissue-reparative M2, a shift that may result from macrophage phagocytosis of debris.^{8–11} Tissue-reparative intramuscle macrophages then proliferate and in turn stimulate myogenesis and fiber growth that lead to muscle repair.⁷ In support of this concept, even partial macrophage depletion in the inflamed muscle impedes regeneration.¹² Taken together, the shift from an M1 cyto-destructive to a dominant M2 tissue-reparative macrophage population is central to muscle repair.

However, macrophage-rich *Dysf*-deficient muscles do not heal, but rather succumb to progressive muscular dystrophy. Is failed muscle repair in *Dysf*-deficient hosts dependent on the absence of this gene in muscles and/or in macrophages? Ample evidence indicates that inflammation is initiated in the *Dysf*-deficient muscle.^{13,14} This concept is based on the central role of *Dysf*, a calcium binding membrane-associated protein, in critical membrane functions, including repair.³ Convincingly, the pathologic process of *Dysf*-deficient (A/J) mice is completely rescued by overexpressing *Dysf* in skeletal muscle (*Dysf* transgenic mice), suggesting that the phenotype of muscular dystrophy is initiated in muscle and not by other cell types.¹⁵ However, once initiated, we hypothesize that macrophages contribute to progressive muscle destruction. To test this hypothesis, we investigated whether i) the *Dysf*-deficient inflamed muscle microenvironment recruits macrophages, skews these myeloid cells toward an M1 macrophage cyto-destructive, not M2 macrophage tissue-reparative, phenotype, and fosters proliferation of cyto-destructive macrophages; ii) the absence of *Dysf* in muscle, but not in macrophages, mediates recruitment into the inflamed muscle and promotes proliferation; and iii) *Dysf*-deficient myogenic cells in comparison with *Dysf*-intact cells are more vulnerable to M1 macrophage-mediated muscle destruction (apoptosis/necrosis) and skew macrophages to a M1 phenotype.

Because the human and mouse *Dysf* genes share >90% amino acid sequence homology,¹⁶ we used the well-characterized *Dysf*-deficient mutant mice that develop progressive dysferlinopathy¹⁷ to probe for macrophage–muscle interactions instrumental in this disease. We tracked macrophage phenotypes and actions within inflamed muscle using genetic fate mapping. Coupled with *in vitro* strategies we clarified macrophage–muscle interactions central to dysferlinopathy. Combined, our *in vivo* and *in vitro* data

suggest that *Dysf* deficiency in muscle, not macrophages, drives macrophage recruitment and skewing toward a cyto-destructive phenotype, which may, in turn, lead to muscle destruction. Thereby, our findings offer the potential of identifying novel therapeutic targets for dysferlinopathy.

Materials and Methods

Mice

The transgenic strain (*Tg^{fms}-EGFP*), expressing enhanced green fluorescent protein (eGFP) under control of the colony-stimulating factor 1 receptor (*c-fms*) promoter and first intron (referred to as MacGreen mice), were provided by Dr. David Hume (Roslin Institute, Edinburgh, Scotland). These mice were backcrossed into the B6 background.^{18,19} *Dysf*-deficient B6.A-*Dysf*^{prmd}/GeneJ (BLA/J), MacGreen reporter mice on the BLA/J background B6.A-*Dysf*^{prmd}/GeneJxB6N.Cg-Tg (Csf-1r-EGFP)1Hume/J (MacGreen: BLA/J), and C57Bl/6 (B6) background were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were housed at Harvard Medical School. Use of mice in this study was reviewed and approved by the Standing Committee on Animals in the Harvard Medical School in adherence to standards set in the *Guide for the Care and Use of Laboratory Animals*.²⁰

Muscle Histopathologic Analysis

Muscle tissues for histopathologic analysis were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned (5 μ m), and stained with hematoxylin and eosin. Muscle disease was assessed according to the following scoring system: 0 = normal muscle with peripheral nuclei, tightly and orderly aligned fibers of relatively the same size; 1 = signs of muscle damage, including necrosis, cell infiltration, regenerating fibers, fiber size variation, no or little fat/adipose tissue deposition; 2 = same signs as score 1 with increased fiber size variation and moderate fat deposition ($\leq 50\%$); 3 = extensive degree for all signs of muscle injury and extensive adipose tissue deposition ($\geq 50\%$).

Immunostaining

Muscle cryosections (5 μ m) were stained for the presence of macrophages, using anti-mouse anti-F4/80 antibody (Ab; BM-8; Invitrogen, Carlsbad, CA) and anti-mouse CD68 Ab (FA-11; Bio-Rad AbD Serotec, Raleigh, NC), as previously detailed.¹⁸ To combine immunostaining with histopathologic analysis, we counterstained with hematoxylin and eosin. To determine the number of proliferating macrophages, we stained cryosections of muscle with anti-mouse CD68 Ab, and anti-mouse Ki-67 Ab (SP6; Vector Laboratories, Burlingame, CA), followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-rat IgG Ab (Invitrogen) and cyanine (Cy)3-conjugated goat anti-rabbit IgG Ab

(Invitrogen). We enumerated the number of CD68⁺/Ki-67⁺ cells per field in the entire cross section.

Flow Cytometry

Muscle tissues were minced and dissociated into a single-cell suspension using collagenase type 4 (1 mg/mL; Worthington, Lakewood, NJ). We stained single-cell suspensions from muscles or blood cells as described previously.²¹ To detect intracellular antigens, we stained the extracellular antigens for 20 minutes at 4°C. Then, we fixed cells with 4% paraformaldehyde at room temperature for 20 minutes²² and washed once in permeabilization wash buffer (BioLegend, San Diego, CA). Subsequently, we incubated the cells with Abs for intracellular antigens in permeabilization wash buffer overnight at 4°C. After washing, cells were counted and analyzed on BD LSR II (BD Biosciences, San Jose, CA) equipped with a high-throughput screening unit.

Flow Cytometric Abs

We used the following Abs from BioLegend for fluorescence-activated cell sorting analysis: FITC or Brilliant Violet 510-conjugated anti-mouse/human CD11b Ab (M1/70), Pacific Blue-conjugated anti-mouse CD45 Ab (30-F11), phycoerythrin (PE)/Cy7-conjugated anti-mouse IL-10 Ab (JES5-16E3), biotinylated or PE-conjugated anti-mouse Ly6C Ab (HK1.4), allophycocyanin/Cy7-conjugated anti-mouse Ly6G Ab (1A8), allophycocyanin-conjugated anti-mouse transforming growth factor (TGF)-β1 Ab (TW7-16B4), peridinin chlorophyll protein/Cy5.5-conjugated anti-mouse tumor necrosis factor (TNF)-α Ab (MP6-XT22), and peridinin chlorophyll protein/Cy5.5-conjugated streptavidin. In addition, we used FITC-conjugated anti-nitric oxide synthase type II Ab (NOS-II; 6/iNOS/NOS Type II) from BD Biosciences, PE-conjugated anti-NOS-II Ab (CXNFT) from eBioscience (San Diego, CA), and PE-conjugated anti-Arginase-1 polyclonal AB from R&D Systems (Minneapolis, MN).

BrdU Incorporation Assay

Unless otherwise stated, we injected mice with bromodeoxyuridine (BrdU; 2 mg/mouse i.p.; Sigma-Aldrich, St. Louis, MO) 3 hours before sacrifice. BrdU⁺ cells were analyzed by flow cytometry with an anti-BrdU Ab (Bu20a; BioLegend).

Adoptive Transfer

Bone marrow (BM) was isolated from MacGreen:BLA/J or MacGreen:B6 mice and adoptively transferred these eGFP⁺ cells (1×10^8 , intravenously injected) into BLA/J and B6 mice. Mice were sacrificed and muscle and blood samples were processed to detect eGFP⁺ cells using flow cytometry as previously described.²²

Thioglycollate-Induced Peritonitis

Peritonitis was induced using thioglycollate (1.5 mL of 4%) injected (i.p.) into BLA/J and B6 mice (11 weeks of age). At varying times after injection, peritoneal cells were harvested and analyzed by flow cytometry.

Myogenic Cell—Mediated Macrophage Skewing

Dysf-deficient (A/J) and wild-type (WT) myoblasts were a kind gift from Dr. Terence Partridge (Children's National Medical Center, Washington, DC). Cells were grown as previously described.^{23,24} On reaching confluence, myoblasts were induced to differentiate into myotubes (single to triple nucleated myocytes; referred to as myogenic cells) for 5 days with a media change at day 2, in myotube differentiation media [Dulbecco's modified Eagle's medium (DMEM) supplemented with 2% horse serum]. Cell-free myogenic cell—conditioned medium was collected at day 5 for macrophage stimulation. WT eGFP⁺ BM macrophages were generated from mouse BM as previously detailed.²¹ Before stimulation, BM macrophages were plated overnight in L929 media. The next day, cells were primed overnight with 100 IU/mL interferon (IFN)-γ in RPMI media containing 10% fetal calf serum (Gibco, Carlsbad, CA). After priming, cells were washed and stimulated with myogenic cell—conditioned media supplemented with 5 μg/mL Brefeldin A (BioLegend). Control cells were treated with neat myotube differentiation media supplemented with 100 ng/mL lipopolysaccharide (LPS). After 3.5 hours of stimulation, cells were harvested using Cellstripper (CellGro, Manassas, VA). Extracellular (F4/80) and intracellular staining was performed as described above, with intracellular staining for 1 hour, before washing and fluorescence-activated cell sorting analysis.

Macrophage-Mediated Myogenic Cell Apoptosis

Dysf-deficient and WT myoblasts were induced to differentiate at approximately 70% confluence for 2 days. Subsequently, 3×10^5 WT eGFP⁺ BM macrophages were added per well in myotube differentiation media. BM macrophages for co-culture experiments were stimulated for 45 minutes with 20 ng/mL TNF-α and 100 ng/mL LPS at 37°C in RPMI supplemented with 10% fetal calf serum (Gibco).

Induction of Macrophage Proliferation by Myogenic Cell Supernatant Fluid

Myogenic cells were stimulated with 25 ng/mL murine TNF-α (R&D Systems) for 24 hours. Cells were washed three times with phosphate-buffered saline and further cultured in serum-free medium. After an additional 24 hours, cell supernatant fluid was collected and used to stimulate BM macrophages starved in DMEM without L929 for 2 hours. These BM macrophages were incubated with

myogenic cell supernatant fluids for 24 hours. Macrophage proliferation was analyzed using 1 mmol/L BrdU incorporation for 2 hours and flow cytometric analysis.

Statistical Analysis

Data represent the means $\pm$ SEM prepared using GraphPad Prism software version 5.0 (GraphPad Software, La Jolla, CA). Normality was tested using the Shapiro–Wilk test. Data were analyzed using the nonparametric *U*-test to evaluate *P* values. *P* < 0.05 was considered significant.

Results

Tissue-Resident Macrophages Progressively Accumulate in Proportion to the Severity of Dysferlinopathy in BLA/J Mice

The severity of the pathologic disorder is highly variable in patients with dysferlinopathy.^{17,25} In general, muscles in the limb-girdle region are most susceptible to this illness.^{1,26} With the use of BLA/J mice [A/J mice spontaneously deficient in *Dysf* backcrossed into the C57BL/6 (B6) background],²⁵ we systematically analyzed the magnitude of the disease in different muscle groups: the psoas, gluteus, and tibialis anterior (TA). Muscle pathologic characteristics consisted of increased inflammatory infiltrates surrounding necrotic myofibers and adipocyte infiltration with age (Supplemental Figure S1A). Consistent with prior reports,^{17,25} disease was more prominent in the psoas muscle than in the gluteus muscle, whereas the TA muscle remained largely unaffected, even at 12 months of age (Supplemental Figure S1B). Therefore, to test our hypothesis, we used the gluteus and psoas muscles with advancing dysferlinopathy and the TA as an unaffected control.

To quantify the progressive rise in myeloid cells in muscle during dysferlinopathy, we probed for macrophages and neutrophils in the gluteus, psoas, and TA muscles during advancing disease (Supplemental Figure S2A). Macrophages (CD45⁺CD11b⁺Ly6G[−]F4/80⁺) were the predominant leukocyte population and increased with advancing stages of dysferlinopathy (Supplemental Figure S2A and Supplemental Figure S3). Macrophages were more abundant in the psoas than gluteus muscles (8 months of age); however, macrophages continued to rise in the gluteus but leveled off in the psoas (12 to 14 months of age) owing to massive tissue destruction (Supplemental Figure S3). By comparison, macrophages rose at a much slower rate in the TA muscle (Supplemental Figure S3). To pinpoint the location of intramuscle macrophage-advancing dysferlinopathy, we probed for macrophage expression *in situ*. The majority (approximately 85%) of macrophages (F4/80⁺) accumulated adjacent to necrotic muscle fibers in the *Dysf*-deficient gluteus muscle (BLA/J mice 14 months of age) (Supplemental Figure S2B). Thus, macrophages

accumulated adjacent to necrotic myofibers in BLA/J mice with advancing dysferlinopathy.

To establish the origin of macrophages in BLA/J muscle, we probed for Ly6C expression, a surface marker expressed by blood circulating inflammatory monocytes recruited into inflamed tissues.⁷ In contrast, tissue-resident macrophages are Ly6C[−]. We found that most macrophages in inflamed *Dysf*-deficient gluteus muscle were Ly6C[−] and these macrophages increased with advancing disease (Supplemental Figure S2A). These results suggested that either the bulk of intramuscle macrophages arise from local proliferation of tissue-resident macrophages or recruited Ly6C⁺ monocytes were converted into Ly6C[−] macrophages within the *Dysf*-deficient inflamed microenvironment.⁷ In contrast, neutrophils (CD45⁺CD11b⁺Ly6G⁺), acute responders to muscle injury, were barely detectable at any age (Supplemental Figure S2A). These results indicated that Ly6C[−] macrophages comprise the predominant myeloid population that increase with advancing disease in inflamed BLA/J muscle.

Circulating Monocytes Are Actively Recruited into *Dysf*-Deficient Muscle with Advancing Disease

To determine whether recruited BM monocytes give rise to macrophages in *Dysf*-deficient muscles, we used a genetic fate mapping approach. We adoptively transferred BM cells from MacGreen:B6 (eGFP reporter for *c-Fms* expressed by macrophages)²⁷ into BLA/J mice. We analyzed adoptively transferred eGFP⁺ BM-derived macrophages (BM macrophages) recruited into the psoas, gluteus, and TA muscles at time-related stages of dysferlinopathy: early (2 months of age), mild (7 months of age), and advanced (10 months of age) (Figure 1A). To identify macrophages that are recruited into muscle, we included F4/80 as a marker. Note, F4/80 was expressed at low levels in circulating monocytes but increased after entry into inflamed tissue (within 3 hours) (Supplemental Figure S4A). Monocyte migration increased in the psoas and gluteus muscles with advancing disease (10 > 7 > 2 months of age) in BLA/J mice. We detected the largest number of eGFP⁺ donor-derived macrophages in the muscle with the most severe disease, the psoas. By comparison, monocyte migration into the TA, a muscle that remained nearly unaffected, barely rose above baseline (B6 mice at 2 months of age) (Figure 1A). These results indicated that monocyte recruitment into inflamed *Dysf*-deficient muscle increases with advancing disease.

Dysf Expression in Monocytes Does Not Alter Recruitment into *Dysf*-Deficient Muscle

Because *Dysf* is expressed in monocytes,^{28,29} we investigated whether recruitment into BLA/J inflamed muscles depends on the absence of *Dysf* in macrophages. We compared the number of intramuscle donor MacGreen:BLA/J (eGFP⁺ *Dysf*-deficient) and MacGreen:B6 (eGFP⁺ *Dysf*-intact) macrophages after adoptive transfer

Macrophages Proliferate Adjacent to Necrotic Muscle Fibers and Thereby May Contribute to Macrophage Accumulation in *Dysf*-Deficient Muscle

Our data indicated that adoptively transferred BM cells are recruited into the *Dysf*-deficient inflamed muscle. However, we found Ly6C⁻, not Ly6C⁺, a marker for migratory inflammatory monocytes, in inflamed muscle that rose with

Figure 2 Macrophage (MΦ) proliferate in BLA/J muscles with dysferlinopathy. Cryosections from gluteus muscles of BLA/J and B6 mice (14 months of age) stained with DAPI (blue), anti-CD68 (green), and anti-Ki67 (red) antibody (Ab). The boxed area is shown at higher magnification in the inset. Arrows indicate proliferating MΦ (Ki-67⁺CD68⁺). Graphs indicate proliferating MΦ (Ki-67⁺CD68⁺)/field in section of entire gluteus and MΦ (%) among proliferating cells (Ki-67⁺). Statistics analyzed with *U*-test. Data are expressed as means ± SEM. *n* = 7. ***P* < 0.01. Original magnification: ×10 (BLA/J); ×40 (inset); ×40 (B6).

advancing dysferlinopathy. Therefore, we hypothesized that the *Dysf*-deficient muscle microenvironment promotes the expansion of macrophages. To test this hypothesis we probed for macrophages dividing within *Dysf*-deficient inflamed BLA/J muscles. With the use of immunostaining, we detected proliferating macrophages (Ki-67⁺CD68⁺) in BLA/J muscles with dysferlinopathy (Figure 2). Moreover, most proliferating cells were macrophages (Figure 2). By comparison, we detected few proliferating macrophages in healthy muscle (Figure 2). The bulk of macrophages in inflamed BLA/J muscle were adjacent to necrotic muscle fibers (Supplemental Figure S2C). Therefore, our data suggested that macrophages adjacent to necrotic muscle fibers are proliferating.

Monocytes Recruited into *Dysf*-Deficient Muscle Are Triggered to Proliferate

To determine whether the monocytes recruited into the inflamed *Dysf*-deficient muscle are triggered to proliferate and thereby account for macrophage expansion, we adoptively transferred MacGreen BM cells (eGFP expression on macrophages) and analyzed macrophage proliferation in the psoas, gluteus, and TA muscles. We used BLA/J mice at 10 to 12 months of age with inflamed muscles as recipients (Figure 3A). Note, monocyte recruitment into BLA/J mice at 2 months of age with normal muscles was minimal (Figure 1A). We analyzed the number of macrophages after 3 hours and 5, 8, and 30 days after adoptive transfer. Because macrophages divide every 18 to 24 hours,³⁰ the number of intramuscle eGFP⁺ macrophages at 3 hours was due to recruitment, whereas eGFP⁺ cells at later points represented macrophages that were recruited and locally multiplied. As expected the donor eGFP⁺ monocytes in the blood were maximal at 3 hours after adoptive transfer and declined with time (Figure 3A). The number of eGFP⁺ donor-derived macrophages in BLA/J muscle (3 hours) increased dramatically by 5 days. As anticipated, eGFP⁺ cells accumulated more in the psoas muscle than the gluteus and the TA muscles, which was sparsely populated by

eGFP⁺ cells. eGFP⁺ macrophages began to decline by 8 days and were no longer detectable by 30 days (Figure 3A). These results suggested that in the long term there is a balance between macrophage proliferation and the disappearance of eGFP⁺ macrophages related to cell death or migration from muscle into draining lymph nodes.³¹ Similarly, CD68 and Ki-67 costaining of cryosections from BLA/J muscles with dysferlinopathy showed that macrophages proliferate within inflamed muscles (Figure 2). Because we could not exclude the possibility that the increase of eGFP⁺ macrophages in muscle, at least partly, resulted from continued recruitment from blood, we administered BrdU to BLA/J mice every 12 hours for 8 days after adoptive transfer. We found that BrdU was incorporated in >93% of eGFP⁺ donor-derived macrophages in the three analyzed muscles at 8 days, but not 3 hours, after adoptive transfer. Note, we found an increase of BrdU⁺ eGFP⁺ cells from 3 hours to 8 days, indicating that host along with donor macrophages are proliferating (Figure 3B). Together, these data suggested that after BM recruitment into the inflamed muscle, local macrophage proliferation leads to an accumulation of macrophages that is proportional to the extent of muscle disease.

Dysf Deficiency in Muscle, Not Macrophages, Regulates Macrophage Proliferation in Dysferlinopathy

To determine whether *Dysf* deficiency in macrophages altered macrophage proliferation in inflamed *Dysf*-deficient muscles, we compared the number of proliferative donor MacGreen:BLA/J and MacGreen:B6 macrophages after adoptive transfer into BLA/J recipients at 10 to 12 months of age (Figure 3C). We detected similar numbers of eGFP⁺ macrophages in the inflamed *Dysf*-deficient muscle (psoas) of MacGreen:BLA/J and MacGreen:B6 BM cells at 3 hours and 5 and 8 days after adoptive transfer (Figure 3C). These data suggested that the *Dysf*-deficient muscle microenvironment, and not the absence of *Dysf* in macrophages, regulated the rate of macrophage proliferation and accumulation in dysferlinopathy.

Figure 3 Recruited macrophage (MΦ) proliferate in response to *Dysf*-deficient myofibers in BLA/J mice. **A:** Enhanced green fluorescent protein (eGFP)⁺ *Dysf*-intact bone marrow (BM) was adoptively transferred into BLA/J mice (10–12 months of age). Donor cell number in the blood and muscles was analyzed at 3 hours and 5, 8, and 30 days after adoptive transfer (AT). **B:** Bromodeoxyuridine (BrdU) incorporation by recruited MΦs in muscles at 3 hours and 8 days after AT of MacGreen:B6 BM cells into BLA/J mice at 10 to 12 months of age. We administered 2 mg of BrdU at the time of AT and then every 12 hours. **C:** Comparison of donor eGFP⁺ *Dysf*-deficient with intact donor MΦ number in three muscles. No significant difference was found between the two groups. **D:** BrdU⁺ BM MΦs stimulated with supernatant fluids from *Dysf*-deficient (A/J) and intact [wild-type (WT)] myogenic cells analyzed by flow cytometry. **Dashed line** (baseline) indicates BM MΦ cultured in serum-free Dulbecco's modified Eagle's medium (DMEM). Statistics analyzed with *U*-test. Data are expressed as means ± SEM. *n* = 4 (**B**); *n* = 3 to 7 (**C**); *n* = 3 to 4 (**D**). **P* < 0.05, ***P* < 0.01. MFI, median fluorescence intensity; TA, tibialis anterior; TNF, tumor necrosis factor.

Dysf-Deficient Myogenic Cells Promote Macrophage Proliferation *in Vitro*

To test the hypothesis that *Dysf*-deficient myotubes directly promote macrophage proliferation, we used an *in vitro* approach (Figure 3D). Therefore, we differentiated myotubes from *Dysf*-deficient (A/J) and *Dysf*-intact (WT) myoblasts (single to triple nucleated myocytes; referred to as myogenic cells). Then, we analyzed macrophage proliferation after exposure to *Dysf*-deficient and -intact (TNF- α stimulated) myogenic cell-conditioned media. Supernatant fluid (neat) derived from *Dysf*-deficient, but not *Dysf*-intact myogenic cells, induced macrophage proliferation (Figure 3D). Note, macrophages stimulated with L929 and fetal calf serum and media alone (DMEM) served as

positive and negative controls, respectively. Taken together, these data suggested that *Dysf*-deficient muscle directly promotes intramuscle macrophage proliferation.

Intramuscle Proliferating Macrophages Display a Cyto-Destructive Phenotype in Dysferlinopathy

Because macrophage-rich *Dysf*-deficient muscles progressively deteriorated with time, we hypothesized that the accumulating macrophages are skewed toward a cyto-destructive phenotype. To test this hypothesis, we probed for cyto-destructive (M1) and tissue-reparative phenotypes (M2) during advancing stages of dysferlinopathy in BLA/J mice: before (1.5 months of age), mild (8 months of age), and advanced (14 months of age). We probed for the

Figure 4 Proliferating macrophages (M Φ) in BLA/J muscles express cyto-destructive mediators, rather than tissue-reparative cytokines. **A:** M Φ s isolated from gluteus muscles of BLA/J mice (8 and 14 months of age) stained for proinflammatory and tissue-reparative cytokines evaluated by flow cytometry. Graphs represent M Φ s expressing classically activated (M1) and alternatively activated (M2) markers in gluteus muscles of BLA/J mice during advancing age. **Dashed lines** indicate M Φ s in gluteus muscles of B6 mice (8 months of age). **B:** Analysis of proliferating M Φ s (BrdU $^{+}$) in BLA/J mice (3 hours) after injecting BrdU. **C:** eGFP $^{+}$ *Dysf*-deficient and -intact bone marrow (BM) (1×10^8) were adoptively transferred into BLA/J recipients (10 months of age). Polarization of donor M Φ s was analyzed (5 days later) in the psoas, gluteus, and tibialis anterior (TA) muscles using flow cytometry. Statistics analyzed with *U*-test. Data are expressed as means $\pm$ SEM. $n = 5$ to 7 (**A**); $n = 7$ (**B**); $n = 6$ (**C**). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Arg-1, arginase-1; BrdU, bromodeoxyuridine; eGFP, enhanced green fluorescent protein; NOS-II, nitric oxide synthase type II; TGF, transforming growth factor; TNF, tumor necrosis factor.

expression of functional molecules secreted from macrophages that mediate tissue injury (TNF- α ,⁷ NOS-II³²) and repair (TGF- β ,³³ IL-10,^{32,34} and Arginase-1³⁴). We detected more cyto-destructive (TNF- α and NOS-II) compared with tissue-reparative (TGF- β , IL-10, and Arginase-1) markers with advancing disease (Figure 4A and Supplemental Figure S4B). By comparison, B6 intramuscle macrophages were far less skewed toward an M1 phenotype than age-matched BLA/J mice (Figure 4A). Note, although co-expression of macrophage markers added depth to phenotype analysis, our methods were restricted to single markers. Taken together, our data indicated that most macrophages in *Dysf*-deficient muscle express a cyto-destructive rather than tissue-reparative phenotype.

To quantify proliferating cyto-destructive and tissue-reparative macrophage phenotypes during advancing dysferlinopathy, we injected BrdU (i.p.) and analyzed intramuscle macrophage expression of these phenotypes (after 3 hours) in mice at 8 and 14 months of age (Figure 4B). We determined that proliferating macrophages (BrdU⁺) predominantly expressed cyto-destructive compared with tissue-reparative macrophage markers in the gluteus muscle (Figure 4B). These data indicated that an expansion of M1 macrophages is associated with advancing dysferlinopathy.

To determine whether the absence of *Dysf* in macrophages promoted skewing toward a cyto-destructive macrophage phenotype, we compared the magnitude of cyto-destructive with tissue-reparative macrophages in adoptively transferred BM cells from MacGreen:BLA/J and MacGreen:B6 mice. Moreover, we quantified cyto-destructive and tissue-reparative donor eGFP⁺ macrophages in muscle groups (psoas, gluteus, TA) with varying degrees of disease (Figure 4C). We detected similar numbers of eGFP⁺ cyto-destructive and tissue-reparative macrophages in the inflamed muscle after adoptive transfer. Thus, macrophage *Dysf* expression did not alter cyto-destructive or tissue-reparative macrophage accumulation in *Dysf*-deficient muscle.

Dysf-Deficient Myogenic Cells Promote Cyto-Destructive Macrophage Phenotypes *in Vitro*

Cytokine expression is intrinsically elevated in *Dysf*-deficient myofibers.^{23,29} Therefore, we hypothesized that *Dysf*-deficient myogenic cells express molecules that skew macrophages toward a cyto-destructive phenotype. To test this hypothesis, we stimulated IFN- γ -primed B6 macrophages with conditioned media from A/J and WT myogenic cultures at day 5 of differentiation (Figure 5A). *Dysf*-deficient, but not *Dysf*-intact, conditioned media promoted the expression of cyto-destructive macrophage markers (TNF- α and NOS-II), similar to that induced by LPS stimulation (Figure 5A). By comparison, conditioned media from WT cultures increased macrophage tissue-reparative phenotype (TGF- β). Together, these data indicated that *Dysf*-deficient myogenic cultures promote a cyto-destructive macrophage phenotype.

Cyto-Destructive Macrophages Promote Apoptosis/Necrosis in *Dysf*-Deficient Myogenic Cells *in Vitro*

We identified more proliferating cyto-destructive compared with tissue-reparative macrophages accumulating in *Dysf*-deficient inflamed muscle. Moreover, cyto-destructive macrophages progressively accumulated with advancing dysferlinopathy. Notably, most cyto-destructive macrophages in BLA/J muscle were adjacent to necrotic myofibers (Supplemental Figure S2C). Thus, we hypothesized that proliferating cyto-destructive macrophages promoted myofiber damage and thereby contributed to advancing dysferlinopathy. To determine whether cyto-destructive macrophage induced muscle death (apoptosis, necrosis), we compared macrophage-mediated apoptosis (Annexin V) and necrosis (propidium iodide) in *Dysf*-deficient and -intact myogenic cultures. *Dysf*-deficient and -intact myogenic cultures were stimulated with M1 (TNF- α , NOS-II expressing) macrophage supernatant fluid (24 hours) (Figure 5B). M1 macrophages induced apoptosis and necrosis in *Dysf*-deficient and -intact myogenic cultures (Figure 5B). Importantly, *Dysf*-deficient myogenic cultures were more susceptible to macrophage-mediated apoptosis and necrosis than *Dysf*-intact myogenic cell cultures (Figure 5B). Taken together, our findings indicated that *Dysf*-deficient compared with *Dysf*-intact myogenic cells were more vulnerable to M1 macrophage-mediated muscle destruction, at least *in vitro*.

From our findings, we propose that the following are key steps leading to dysferlinopathy (Figure 6): i) intrinsic defective muscle repair (the *Dysf*-deficient muscle microenvironment), ii) macrophage recruitment from BM, iii) intramuscle macrophage proliferation, iv) intramuscle macrophage polarization toward a M1 cyto-destructive phenotype, and v) macrophage-mediated muscle apoptosis/necrosis.

Discussion

We tested the hypothesis that *Dysf*-deficient muscle promotes recruitment, proliferation, and skewing of macrophages toward a cyto-destructive phenotype in dysferlinopathy. With the use of *in vivo* strategies, most notably genetic fate mapping, coupled with *in vitro* macrophage and myogenic cell interactions, we report the following novel findings: i) the *Dysf*-deficient muscle microenvironment recruits macrophage, skews these myeloid cells toward an M1 macrophage cyto-destructive, not an M2 macrophage tissue-reparative, phenotype, and fosters proliferation of cyto-destructive macrophage; ii) intramuscle macrophage recruitment and proliferation increases with advancing dysferlinopathy; iii) *Dysf*-deficient compared with *Dysf*-intact myogenic cultures are more vulnerable to M1 macrophage-mediated muscle destruction (apoptosis/necrosis); and iv) the absence of *Dysf* in muscle, but not in macrophages, mediates macrophage recruitment into the inflamed muscle, promotes macrophage proliferation, likely leading to destruction. Taken together,

Figure 5 *Dysf*-deficient myogenic cells promote classically activated (M1) macrophage (MΦ) skewing which induces myotube apoptosis/necrosis *in vitro*. **A:** MΦs primed with interferon (IFN)-γ and subsequently stimulated with myogenic cell-conditioned media. Cells were harvested for intracellular staining using flow cytometry. Representative fluorescence-activated cell sorting (FACS) plots of M1 and alternatively activated (M2) MΦ skewing. Graphs are MΦ skewing in response to myogenic cell-conditioned media. **B:** *Dysf*-deficient and wild-type (WT) myoblasts induced to differentiate at approximately 70% confluence. MacGreen MΦs suspended in myotube differentiation media were added to myogenic cultures after stimulation. After 24 hours of co-culture, cells were harvested and stained with Annexin V (AV) and propidium iodide (PI). Flow cytometry was performed on myogenic cells identified as enhanced green fluorescent protein (eGFP)⁺ cells. Graphs are early apoptotic (AV⁺PI⁻), apoptotic/necrotic (AV⁺PI⁺), and necrotic (AV⁻PI⁺) cells. Statistics analyzed with *U*-test. Data are expressed as means ± SEM. *n* = 3 (**A**); *n* = 3 to 4 (**B**). **P* < 0.05. Arg-1, arginase-1; BM bone marrow; FITC, fluorescein isothiocyanate; FSC, forward scatter; LPS, lipopolysaccharide; NOS-II, nitric oxide synthase type II; TGF, transforming growth factor; TNF, tumor necrosis factor.

Figure 6 Macrophage (MΦ)-mediated muscle destruction during dysferlinopathy. We propose key steps leading to dysferlinopathy: 1) intrinsic defective muscle repair, the *Dysf*-deficient muscle microenvironment, 2) recruits MΦ from bone marrow, 3) fosters MΦ proliferation, 4) polarizes MΦ toward an classically activated (M1) cyto-destructive phenotype which in turn, and 5) releases mediators that induce apoptosis/necrosis and thereby destroy muscle.

although dysferlinopathy is most likely intrinsically initiated in muscle, our data suggest that the influx and expansion of cyto-destructive macrophage phenotypes may contribute to advancing disease.

Macrophage accumulation in *Dysf*-deficient muscle is a hallmark for dysferlinopathy. However, even in a muscle destined for destruction, there are multiple reasons for intramuscle macrophage accumulation. As in acute injury, intramuscle macrophages may be healers, but repeated, persistent muscle insults may override macrophage-mediated repair and thereby lead to loss of function. We have ruled out this possibility. We report that the *Dysf*-deficient inflamed muscle microenvironment recruits macrophages that are polarized toward a cyto-destructive phenotype and expand locally. These events escalate with age in BLA/J mice and are thereby likely to contribute to unrelenting muscle damage, suggesting that inflamed *Dysf*-deficient muscle generates molecules, such as cytokines and damage-associated molecular patterns, which directly or indirectly polarize and sustain a cyto-destructive macrophage phenotype. One plausible candidate is IL-1 β . For example, stimulated *Dysf*-deficient compared with *Dysf*-intact primary myotubes secrete IL-1 β ,^{23,24,29} which can induce macrophages to express TNF- α ,³⁵ and in turn lead to muscle wasting.³⁶ IFN- γ is another cytokine likely expressed in *Dysf*-deficient myotubes²⁴ and injured muscles,³⁷ which is capable of polarizing macrophages toward an M1 phenotype.³⁸ There are numerous other inflammatory molecules that are expressed in *Dysf*-deficient human muscle³⁹ that may promote cytokine-rich inflammation and, in turn, macrophage skewing in dysferlinopathy. For example, TNF- α may be a useful therapeutic target in dysferlinopathy. TNF- α inhibition attenuates muscle disease in dystrophin deficiency (Duchenne's muscular dystrophy).^{40,41} Further, in mucopolysaccharidoses, an inherited lysosomal storage disease, TNF- α inhibition attenuates cellular apoptosis and disease characteristics.^{42,43} Because *Dysf*-deficient myoblasts display impaired lysosome trafficking,⁴⁴

this further supports the therapeutic utility of inhibiting TNF- α in dysferlinopathy. An ideal therapeutic would convert destroyer to healers, a difficult task to even begin without knowing the precise cocktail of cytokines in the *Dysf*-deficient muscles that polarize macrophages toward cyto-destroyers.

Sterile inflammation originates intrinsically within *Dysf*-deficient muscles, plausibly triggered by impaired membrane sealing in response to muscle damage.^{3,15,45} Faulty muscle repair results in continuous macrophage recruitment into in *Dysf*-defective injured muscles. With the use of genetic fate mapping to track macrophages, we now report that macrophage recruitment escalates with advancing age in dysferlinopathy in a muscle-specific pattern. Chemokines, such as monocyte chemoattractant protein 1, known to draw monocytes into tissues, up-regulated in *Dysf*-deficient hosts,²⁴ are likely candidates responsible for recruiting macrophages into inflamed muscles.⁴⁶ For example, monocyte chemoattractant protein 1 and CCR2 are up-regulated in *Dysf*-deficient muscles, and targeting CCR2 results in improved symptoms and function.^{24,47} Moreover, mobilization of macrophages is impaired from BM to blood and blood to injured muscle in CCR2 knockout mice.⁴⁸ We show that WT macrophages are recruited into the *Dysf*-deficient microenvironment. These data suggest that *Dysf*-deficient muscle is responsible for drawing macrophages into the injured muscle before skewing these myeloid cells toward a cyto-destructive phenotype.

Because macrophage express *Dysf*, a central issue has been whether the absence of *Dysf* in macrophages, in addition to muscle, regulates macrophage action in the pathogenesis of dysferlinopathy. With the use of genetic fate mapping to track macrophages with and without *Dysf* expression, we show that the *Dysf*-deficient muscle, independent of macrophage *Dysf* expression, recruits and fosters intramuscle macrophage proliferation. Consistent with this finding, *Dysf*-intact BM transplanted into *Dysf*-deficient mice does not improve muscle function.¹⁴ Within the

context of gene therapy and based on our findings, restoration of *Dysf* expression in muscle, not macrophages, is likely necessary for therapeutic benefit.

There are several explanations as to why *Dysf*-deficient compared with *Dysf*-intact muscle is more vulnerable to macrophage-mediated apoptosis/necrosis. *Dysf* is a key protein in muscle membrane repair and vesicle trafficking⁴⁹; thus, the absence of *Dysf* increases muscle susceptibility to injury. More specifically, intrinsic impairments in Ca^{2+} regulation likely predispose damage in the context of both contraction and inflammation-induced muscle injury. For example, NOS-II disrupts Ca^{2+} signaling to promote muscle damage in *Dysf*-deficient mice. Moreover, similar to dystrophin-deficient muscle, in the absence of *Dysf* contraction-induced myofiber Ca^{2+} influx likely leads to proteasome activation and protein degradation.^{50–52} Given the vulnerability of *Dysf*-deficient muscle to macrophage-mediated apoptosis/necrosis, we suggest that a reduction in cyto-destructive macrophages within muscle is central to suppressing muscular dystrophy. However, because these observations were made *in vitro*, further *in vivo* experiments are required to validate this hypothesis. In addition, our myogenic cultures contained both myocytes and larger multinucleated myotubes. Use of *ex vivo* single-fiber cultures may help elucidate whether macrophages directly induce myofiber destruction in dysferlinopathy. Further, we did not evaluate the effects of *Dysf*-deficient macrophages on myofiber apoptosis/necrosis because of technical limitations.

Taken together, we report an expansion of cyto-destructive macrophages in muscle that are associated with promoting dysferlinopathy. Because proliferating cyto-destructive macrophages are prominent in inflamed *Dysf*-deficient muscles, suppressing intramuscle macrophage recruitment, proliferation of cyto-destructive macrophage, and/or converting cyto-destructive to tissue reparative macrophages are promising therapeutic approaches for suppressing dysferlinopathy.

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Supplemental Data

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