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Molecular Pathogenesis of Genetic and Inherited Diseases

Dysferlin Deficiency Enhances Monocyte Phagocytosis

A Model for the Inflammatory Onset of Limb-Girdle Muscular Dystrophy 2B

Kanneboyina Nagaraju,* Rashmi Rawat,* Edina Veszelovszky,* Rachana Thapliyal,† Akanchha Kesari,* Susan Sparks,* Nina Raben,‡ Paul Plotz,‡ and Eric P. Hoffman*

From the Research Center for Genetic Medicine, Children's National Medical Center, Washington, District of Columbia; the Department of Medicine,† Division of Rheumatology, The Johns Hopkins University School of Medicine, Baltimore Maryland; and the Arthritis and Rheumatism Branch,‡ National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, Bethesda, Maryland*

Dysferlin deficiency causes limb-girdle muscular dystrophy type 2B (LGMD2B; proximal weakness) and Miyoshi myopathy (distal weakness). Muscle inflammation is often present in dysferlin deficiency, and patients are frequently misdiagnosed as having polymyositis. Because monocytes normally express dysferlin, we hypothesized that monocyte/macrophage dysfunction in dysferlin-deficient patients might contribute to disease onset and progression. We therefore examined phagocytic activity, in the presence and absence of cytokines, in freshly isolated peripheral blood monocytes from LGMD2B patients and in the SJL dysferlin-deficient mouse model. Dysferlin-deficient monocytes showed increased phagocytic activity compared with control cells. siRNA-mediated inhibition of dysferlin expression in the J774 macrophage cell line resulted in significantly enhanced phagocytosis, both at baseline and in response to tumor necrosis factor-α. Immunohistochemical analysis revealed positive staining for several mononuclear cell activation markers in LGMD2B human muscle and SJL mouse muscle. SJL muscle showed strong up-regulation of endocytic proteins CIMPR, clathrin, and adaptin-α, and LGMD2B muscle exhibited decreased expression of decay accelerating factor, which was not dysferlin-specific. We further showed that expression levels of small Rho family GTPases RhoA, Rac1, and Cdc 42 were increased in dysferlin-deficient murine immune cells compared with control cells. Therefore, we hypothesize that mild myofiber damage in dysferlin-deficient muscle stimulates an inflammatory cascade that may initiate, exacerbate, and possibly perpetuate the underlying myofiber-specific dystrophic process. *(Am J Pathol 2008, 172:774–785; DOI: 10.2353/ajpath.2008.070327)*

Limb girdle muscular dystrophy type 2B (LGMD2B) and Miyoshi myopathy, a distal myopathy, are both caused by recessively inherited mutations in the dysferlin gene.[1,2] Both disorders show a loss of dysferlin protein at the plasma membrane in myofibers, which leads to abnormalities in vesicle traffic and membrane repair.[3,4] However, dysferlin is also expressed in many other cell types, including $CD14^+$ monocytes.[5,6] Thus, nonmyofiber cells may possibly contribute to the disease process.

Dysferlin is a C2 domain-containing 230-kDa transmembrane protein, principally localized to the intracellular face of the plasma membrane.[7,8] The dysferlin gene is a large 55-exon gene localized to 2p13.[1,2] Dysferlin was originally thought to play a role in membrane vesicle fusion events through its extensive sequence homology to the *Caenorhabditis elegans* FER-1 protein, which is known to be important for vesicle fusion during spermatogenesis.[9] More recent data regarding isolated myofibers are consistent with a role for dysferlin in membrane

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Address reprint requests to Kanneboyina Nagaraju, D.V.M., Ph.D., Director, Murine Drug Testing Facility, Research Center for Genetic Medicine, Children's National Medical Center, Associate Professor of Pediatrics, The George Washington University Medical Center, 111 Michigan Ave., N.W., Washington, DC 20010. E-mail: knagaraju@cnmcresearch.org.

vesicle trafficking and membrane repair.[10,11] Muscle samples from patients with a dysferlin deficiency show numerous extrastructural membrane defects when analyzed by electron microscopy, including tears in the plasma membrane and an accumulation of subsarcolemmal vesicles and vacuoles.[12]

Although it is clear that there are cell-autonomous membrane abnormalities in dysferlin-deficient myofibers, the presentation and progression of LGMD2B/Miyoshi patients include enigmatic histological and clinical features that are not fully explained by the myofiber defects.[13,14] Patients are typically quite healthy until their late teens, and some patients show athletic prowess at a young age. There are presymptomatic elevations in serum creatine kinase but little evidence of weakness before disease onset. Also, disease onset can be more acute than in other dystrophies, and in some instances it is associated with an environmentally related muscle insult. Finally, muscle biopsies from patients early in the disease process can show striking inflammatory infiltrates in perivascular, perimysial, and endomysial areas of the muscle.[15] In fact, this relatively acute onset and the presence of inflammatory infiltrates often lead to LGMD2B being misinterpreted as polymyositis, an autoimmune disease of muscle.[16]

The inflammatory infiltrates in dysferlin-deficient muscle have been described.[12,14,15,17–19] Both dysferlin-deficient muscle biopsies and polymyositis biopsies often show inflammation; however, there are several differences between the muscle inflammation in dysferlin deficiency and that in other inflammatory muscle diseases.[17] In general, dysferlin-deficient muscle biopsies show approximately twice as many macrophages but half as many $CD8^+$ T cells as in polymyositis.[15] Specifically, the endomysial and perivascular infiltrates in dysferlin-deficient muscle have been reported to consist of $CD4^+$ T cells (40.6 ± 22.8%), macrophages (36.7 ± 23.7%), and $CD8^+$ T cells (11.1 ± 6.6%).[15] Nonnecrotic dysferlin-deficient fibers appear free of all types of infiltrates, suggesting a relative paucity of cytotoxic T-cell-mediated myofiber death, despite the extensive inflammatory cell infiltrates in the muscle. Inflammation is also prominent in other muscular dystrophies, such as fascioscapulohumeral dystrophy, in which a high percentage of B cells and $CD4^+$ T cells are observed in perivascular sites, and in Duchenne dystrophy, in which macrophages and T cells are present, for the most part in necrotic fibers.[20]

Dysferlin is normally expressed in $CD14^+$ monocytes, and $CD14^+$ cells show dysferlin deficiency in LGMD2B and Miyoshi myopathy.[6] Detection of dysferlin in $CD14^+$ cells has become a blood-based test for the diagnosis of dysferlin deficiency, although there have been no published comparisons of the sensitivity and specificity of the muscle biopsy test versus the $CD14^+$ blood test. Efficient membrane regulation is necessary for a number of critical monocyte/macrophage functions, including receptor-mediated phagocytosis, cytokine secretion, and receptor signaling regulation through Rho family small GTPases such as Rac1, RhoA, and Cdc42.[21–24] Given the role of dysferlin in membrane regulation in muscle cells and the presence of dysferlin in monocytes, we hypothesized that dysferlin deficiency may alter the ability of monocytes to perform such functions via small Rho family GTPases. Because monocytes are the precursors to tissue macrophages, we further hypothesized that abnormal macrophage function could play a role in the inflammatory reaction seen in skeletal muscle of many LGMD2B patients.

To test the function of dysferlin-deficient monocytes, we designed a human trial of monocyte function in dysferlin-deficient patients and controls. These findings were then validated *in vivo* in dysferlin-deficient mice (SJL and AJ mouse strains) and *in vitro* in a J774 macrophage cell culture model using siRNA to specifically knock down dysferlin. Our findings suggest that overaggressive signaling in muscle macrophages resulting from dysferlin deficiency in these cells may play a key role in disease onset and progression.

Materials and Methods

Dysferlin-Deficient (LGMD2B, Miyoshi) Patients and Clinical Samples

Samples from 13 dysferlin-deficient patients were used in this study. DNA samples were used for mutation analysis, peripheral blood for phagocytosis assay, and muscle biopsies for immunohistochemistry and mRNA expression profiling. Flash-frozen diagnostic muscle biopsies from dysferlin-deficient patients were received by the Research Center for Genetic Medicine at Children's National Medical Center as part of an ongoing, *gratis* molecular diagnostics program. Biopsies were tested for dystrophin and dysferlin by Western blotting (duplicate blots); immunostaining for merosin (lamina-$\alpha 2$), dystrophin, and α-sarcoglycan; and histological examination (hematoxylin and eosin staining). Biopsies were assigned a tentative diagnosis of primary dysferlin deficiency (LGMD2B, Miyoshi myopathy) if they showed a loss of dysferlin by Western blotting (0 to 10% of normal control levels) but a normal dystrophin Western blot and normal α-sarcoglycan and merosin immunostaining. Muscle biopsies showing complete dysferlin deficiency (0% relative to controls) were then selected for mRNA profiling (patients 1, 2, 4, 7, 10, 11, 12, and 13) and immunohistochemical staining (patients 1, 3, 4, 5, and 6) as described below (Table 1).

The dysferlin gene is difficult to assay for gene mutations, given its large size (55 exons) and the lack of common mutations (most patients have private mutations). We screened DNA samples from all 13 patients for gene mutations by denaturing high-performance liquid chromatography of each dysferlin exon, followed by sequencing of exons containing heteroduplexes by denaturing high-performance liquid chromatography. Pathogenic mutations were detected in 10 of the 13 patients, and a possible exon splicing enhancer mutation was detected in 1 additional patient (patient 6) (Table 1). One of the affected patients was a full sibling to a second mutation-positive patient and therefore was assumed to have the same underlying mutations (patients 2 and 8 in Table 1).

Table 1. Characteristics of Dysferlin-Deficient Patients

No.	Age at biopsy	Sex	Dysferlin	Zygosity	Mutations	Phago	IHC	Microarray
1	19	F	0%	Compound heterozygote	c.610C>T c.4192_4193insC	X	X	X
2	17	M	0%	Compound heterozygote	c.1834C>T splice mut (I-50)	X	nd	X
3.	n/a	F	0%	Compound heterozygote	c.3113G>A c.1749_1750insT	nd	X	nd
4.	31	F	0%	Compound heterozygote	c.2779delG c.4253G>A	nd	X	X
5	27	M	0%	Heterozygous	c.3992G>T	nd	X	nd
6	27	F	0%		ESE detected*	nd	X	nd
7	69	M	0%		None detected	nd	nd	X
8	n/a	M	0%	Compound heterozygote*	c.1834C>T splice mut(I-50)†	X	nd	nd
9	26	M	10%		Not detected	X	nd	nd
10	26	F	0%	Compound heterozygote	c.2643 + 1G>A c.4167 + 1G>C	nd	nd	X
11	23	M	0%	Homozygote	c.5835_5838Del	nd	nd	X
12	37	M	0%	Heterozygote	c.2641A>C	nd	nd	X
13	19	F	0%	Heterozygote	c.3113G>A	nd	nd	X

nd, not done; IHC, immunohistochemistry.
*Exon splicing enhancer (ESE) was detected in exon 20 (c.2197 T>C).
†Patient 8 is a full brother to patient 2 and is assumed to show dysferlin deficiency and have the same mutations, although this was not directly tested.

Under the auspices of an institutional review board-approved protocol conducted through the Children's National Medical Center General Clinical Research Center, we also contacted dysferlin-deficient patients via their referring physicians and invited them to participate in the monocyte study. Four of these patients (patients 1, 2, 8, and 9) volunteered and were brought into the Children's National Medical Center Clinical Research Center for blood draws for monocyte studies using peripheral blood. Four age- and sex-matched healthy adults with normal muscle function were also recruited as controls under the same protocol. All patients gave informed consent.

Dysferlin-Deficient Mouse Model

All animal experiments were conducted in accordance with institutional guidelines. Five-month-old male and female SJL/J (dysferlin-deficient, $n = 16$), AJ/J (dysferlin-deficient, $n = 6$), Mdx (dystrophin-deficient, $n = 6$), and C57BL/6J (normal control, $n = 16$) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were housed in an individually ventilated cage system with a 12-hour light and dark cycle. Blood from tail bleeds of mice was collected in heparinized tubes and stored on ice until assayed. The mice were then euthanized by carbon dioxide gas, and death was ensured by cervical dislocation and verified by checking for a heartbeat. Peritoneal macrophages were collected after euthanasia by injecting 10 ml of ice-cold phosphate-buffered saline (PBS) containing 10% fetal bovine serum into the peritoneal cavity. The recovered peritoneal fluid was washed in chilled PBS. Viability, as determined by trypan blue dye exclusion, was >98%. For some experiments peritoneal macrophages were elicited in C57/BL6 and SJL/J mice and isolated 3 days after the injection of 3% thioglycollate broth. These peritoneal macrophages were washed with Dulbecco's modified Eagle's medium twice and then resuspended in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum at a concentration of 1×10^6/ml and treated with 1 μg/ml of lipopolysaccharide for 4 hours at 37°C in a CO_2 incubator and lysates were prepared as described below. Hind limb muscle tissues were dissected and flash-frozen in isopentane-chilled liquid nitrogen and stored at −80°C until processed for immunohistochemistry and Western blotting.

Immunohistochemical Staining

Immunohistochemical staining was performed on muscle biopsies that had been obtained from five patients (patients 1, 3, 4, 5, and 6) for routine diagnosis at the Research Center for Genetic Medicine. These analyses were used to determine the activation status of the infiltrated macrophages, as described previously.[25,26] Frozen sections of human muscle biopsy samples were stained with mouse anti-human HLA-A, -B, -C (W6/32) (Harlan Sera Labs, Leicestershire, UK), HLA-DR (Tu 36) (BD Pharmingen, Franklin Lakes, NJ), CD86 (IT2.2), or DAF/CD55 (55C02) (Calbiochem, San Diego, CA) antibody. Frozen sections of muscle tissues from SJL/J (dysferlin-deficient) and C57BL/6J (control) mice were stained with rat anti-mouse MoMa-2 (Serotec, Oxford, UK) or with biotinylated anti-mouse CD11c (HL3) or ICAM-1 (3E2) (BD Pharmingen). Anti-mouse horseradish peroxidase, anti-rat horseradish peroxidase, streptavidin-horseradish peroxidase (DAKO, Carpinteria, CA), or anti-mouse IgG Cy™3 (Jackson ImmunoResearch, West Grove, PA) was used as the secondary antibody, as appropriate. Isotype-matched mouse Igs were used instead of primary antibodies as negative controls.

Phagocytic Activity Assays of Human and Mouse Peripheral Blood Monocytes

Phagocytosis was assayed using the Vybrant phagocytosis assay kit from Molecular Probes (Eugene, OR). For human samples, heparinized whole blood was used within 2 hours of collection. Heparinized mouse blood was used within 30 minutes after collection. All materials used for the assay were prechilled on ice for 20 minutes.

Heparinized whole blood (25 μl) was placed in 5-ml tubes, and 75 μl of Hanks' balanced salt solution con-

taining 5% fetal bovine serum was added, along with 10 μl of fluorescent *Escherichia coli* bioparticles (Molecular Probes). The tubes were vortexed and incubated for 10 minutes at 37°C, then transferred to ice, and 100 μl of chilled trypan blue solution was added. The cell suspensions were washed with fluorescence-activated cell sorting buffer (phosphate-buffered saline containing 2% fetal calf serum, 1% bovine serum albumin, and 0.05% sodium azide), and the red blood cells were lysed by the addition of ammonium chloride, potassium chloride lysing solution at room temperature. The remaining cells were washed twice with fluorescence-activated cell sorting buffer, and fluorescence data were collected on 20,000 cells using a FACScan flow cytometer and CellQuest software. Monocyte gating was established using forward and side scatter profiles and further verified by MOMA staining. The phagocytic activity index was calculated by multiplying the percentage of monocytes phagocytosing the fluorescent bioparticles by the mean fluorescent intensity of the monocytes, as determined by CellQuest software. For cytokine incubations, samples were treated with 20 ng/ml of tumor necrosis factor (TNF)-α for 30 minutes at room temperature. All assays were performed using either duplicate or triplicate samples.

Western Blotting

Western blotting of muscle tissue was performed as described previously.[27] Macrophage, spleen, and muscle homogenates were prepared in lysis buffer (1% Nonidet P-40, 20 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1 mmol/L ethylenediaminetetraacetic acid) with 1 mmol/L dithiothreitol and protease inhibitors. Lysates (20 to 100 μg) were electrophoresed on 4 to 12% Bis-Tris Gels (Invitrogen, Carlsbad, CA) and transferred to a nitrocellulose membrane. Immunoblots were performed with antibodies to dysferlin (230 kDa), CIMPR (275 kDa), clathrin (180 kDa), adaptin-α (112 kDa), Cdc-42 (21 kDa), Rac-1 (21 kDa), and RhoA (21 kDa) (Cell Signaling, Beverly, MA). All blots were stripped and blotted for vinculin (117 kDa) or β tubulin (55 kDa) to assess equal gel loading. The autoradiograms were scanned using an Arcus II scanner (Agfa, Mortsel, Belgium) and volume analysis was performed using Quantity one software (Bio-Rad discovery series; Bio-Rad, Hercules, CA). The ratios of CIMPR, clathrin, adaptin-α, Cdc42, Rac-1, and RhoA to vinculin or β-tubulin were calculated for dysferlin-deficient and control mice.

Silencing Dysferlin Expression in the J774 Macrophage Cell Line Using siRNA

The J774 mouse macrophage cell line (TIB67) was obtained from American Type Culture Collection (Rockville, MD) and grown to 60 to 70% confluence in six-well plates in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, then transfected with various concentrations of siRNA oligonucleotides targeting four different sequences within the dysferlin gene (DYSF1, 5′-CCCGAACTATGCCGCCATGAA-3′; DYSF2, 5′-ATCTGTCATCGGAGAATTTAA-3′; DYSF3, 5′-TACCCTGAGCTTTGGCGTTAA-3′; DYSF4, 5′-CAGGATAAGGACTACACCATT-3′) [4 for silencing; Qiagen, Valencia, CA]) using oligofectamine (Invitrogen). Transfection was performed at 50, 100, and 250 nmol/L of control or DYSF siRNA in the presence of serum. We used a cocktail composed of equal concentrations of four siRNAs: DYSF1r (CGAACUAUGCCGCCAUGAAUU) r(UUCAUGGCGGCAUAGUUCGGG), DYSF2 r(CUGUCAUCGGAGAAUUUAAUU) r(UUAAAUUCUCCGAUGACAGAU), DYSF3 r(CCCUGAGCUUUGGCGUUAAUU) r(UUAACGCCAAAGCUCAGGGUA), DYSF4 r(GGAUAAGGACUACACCAUUUU) r(AAUGGUGUAGUCCUUAUCCUG). Negative control siRNA was used at the same total concentration: control r(UUCUCCGAACGUGUCACGU)d(TT)(ACGUGACACGUUCGGAGAA)d(TT). siRNAs were added to 350 μl of OptiMEM (Invitrogen) and incubated for 30 minutes at room temperature. Separately, 5 μl of oligofectamine was mixed with 20 μl of OptiMEM and incubated for 30 minutes at room temperature. The oligofectamine was then mixed with siRNA and incubated for 30 minutes at room temperature. The mixture was overlaid on cells cultured in antibiotic-free medium in six-well plates. After 18 hours, the medium was replaced, and the ability of the cells to phagocytose fluorescent *E. coli* bioparticles was assessed by fluorescence-activated cell sorting analysis as described above. The results were expressed in terms of phagocytosis activity index. Dysferlin expression in these experiments was verified by Western blotting.

mRNA Expression Profiling

The expression profiles used in these studies were from a 128-muscle biopsy, 256-Affymetrix (Santa Clara, CA) profile data set that we described as part of a previously published study of Emery-Dreifuss muscular dystrophy.[28] A full description of the data set can be found in our previous article, although the published data focused on patients harboring emerin and lamin A/C mutations. The present analysis is the first analysis of the data related to dysferlin deficiency (LGMD2B), although the raw data were released to the public before the current publication. Diagnostic groups represented in the data set include normal skeletal muscle ($n = 18$), autoimmune disease ($n = 25$), fukutin-related protein deficiency (missense for glycosylation enzyme; limb-girdle muscular dystrophy 2I; $n = 7$), Duchenne muscular dystrophy (dystrophin deficiency, $n = 10$),[29] Becker muscular dystrophy (partial loss of dystrophin, $n = 5$), dysferlin deficiency (LGMD2B, $n = 8$) (described here), calpain 3 deficiency (LGMD2A, $n = 10$), fascioscapulohumeral dystrophy (FSHD; loss of 4q sequence, $n = 14$), acute quadriplegic myopathy (critical care myopathy, $n = 5$),[30] spastin haploinsufficiency (microtubule traffic defect, $n = 3$),[31] Emery-Dreifuss muscular dystrophy (lamin A/C missense mutations, $n = 4$),[28] Emery-Dreifuss muscular dystrophy X-linked (emerin mutations, $n = 4$),[28] and ALS ($n = 9$). All biopsies were tested in both Affymetrix U133A and U133B arrays, producing 256 sets of microarray results in the data set. Both MAS5.0 and dCHIP mismatch model probe set algorithms were used,

and all profiles are available as both unprocessed data (.dat,.cel files) and processed data in both PEPR (*http://pepr.cnmcresearch.org*) and NCBI GEO.[32] We queried the CD55 gene in the MAS5.0 signals from the data set using the PEPR SAS server interface. The distribution of gene expression level over all classes of muscular dystrophy was graphically represented with box plots.

Statistical Analysis

Test and control samples were compared using Student's *t*-test, and a value of $P < 0.05$ was considered significant.

Results

Macrophages Are Activated Both in Dysferlin-Deficient Patients and SJL/J Mice

An increase in proinflammatory cytokines (ie, interferon-γ) and TNF-α induces macrophage and mononuclear cell activation and maturation. These processes can be identified in humans by measuring the expression of monocyte activation and dendritic cell markers, such as the major histocompatibility complex (MHC) class I (HLA-A, -B, -C) and class II (HLA-DR antigens), ICAM-1, and CD86. Immunohistochemical staining of frozen sections of dysferlin-deficient and control muscle biopsies showed that infiltrating mononuclear and dendritic cells, but not muscle fibers, expressed high levels of HLA-A, -B, -C, HLA-DR, and CD86 antigens (Figure 1, A–C). Low levels of HLA-A, -B, -C and HLA-DR antigen expression were also noted on capillary endothelial cells (Figure 1, A–C). Similarly, staining of frozen muscle sections of SJL/J mice for macrophage activation and dendritic cell markers showed that infiltrating mononuclear cells expressed the macrophage mononuclear cell marker (MoMa-2) and activation markers CD11c and ICAM-1 (Figure 1, D–F). ICAM-1 expression was also noted on some small muscle fibers (Figure 1F). Control muscle biopsy sections and muscle tissue sections from C57BL/6J control mice did not show any infiltration (data not shown). These results suggest that macrophages and monocytes present in the muscle tissues of dysferlin-deficient patients and SJL/J mice have an activated phenotype.

Human and Murine Dysferlin-Deficient Monocytes Show Enhanced Phagocytic Activity

Given the expression of dysferlin in monocytes, we hypothesized that the vesicular trafficking abnormalities seen in muscle might result in abnormalities in macrophage activation and function. Monocytes from control (C57BL/6) and dysferlin-deficient SJL/J mice were therefore tested for their ability to phagocytose fluorescently labeled *E. coli*. The phagocytic activity index was significantly ($P < 0.002$) higher in SJL/J mice (mean $\pm$ SE, 107,038 $\pm$ 16,919) than in age- and sex-matched control C57BL/6J mice (mean $\pm$ SE, 35,293 $\pm$ 11,856) (Figure 2A). We also assessed the phagocytic activity of peritoneal macrophages from these two strains and found that dysferlin-deficient SJL/J mice showed significantly higher

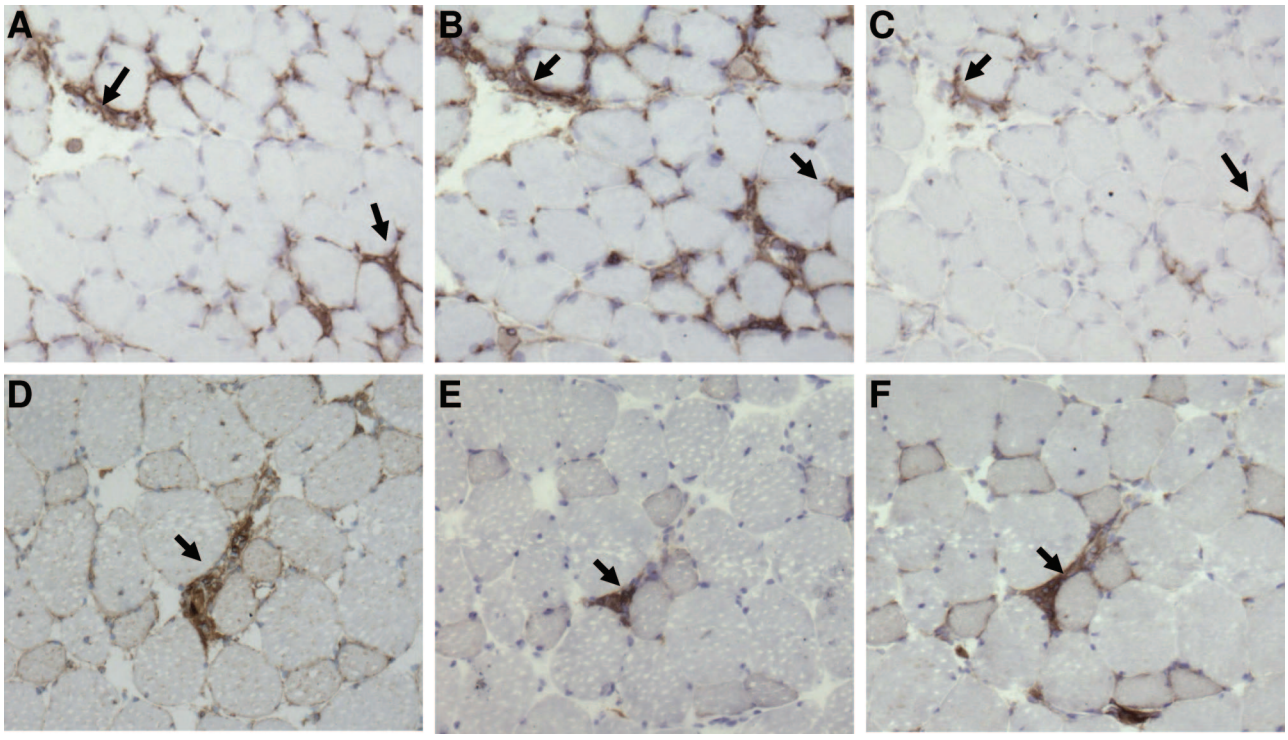

Figure 1. Macrophages are activated in both dysferlin-deficient patients and SJL/J mice. Representative examples of consecutive frozen muscle biopsy sections from dysferlin-deficient patients, stained for the mononuclear cell activation markers HLA-DR (**A**), HLA-A, -B, -C (**B**), and CD86 (**C**). Representative examples of consecutive frozen muscle tissue sections from an SJL/J mouse, similarly stained for the macrophage activation markers MOMA-2 (**D**), CD11c (**E**), and ICAM-1 (**F**).

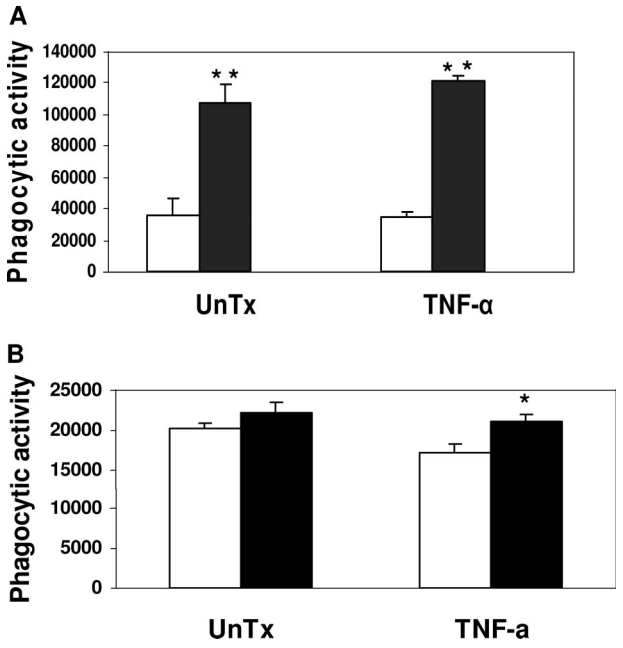

Figure 2. Phagocytic activity of mouse and human dysferlin-deficient monocytes. **A:** Peripheral blood monocytes from control C57BL/6J (**open bars**) and dysferlin-deficient SJL/J mice (**filled bars**) were tested for their phagocytic function in the absence or presence of TNF-α. **B:** Phagocytic activity of human peripheral blood monocytes from normal control individuals (**open bars**) and dysferlin-deficient LGMD2B patients ($n = 4$, **filled bars**) in the absence or presence of TNF-α. $^{**}P \leq 0.001$; $^{*}P \leq 0.05$.

phagocytic activity (mean ± SE, 671,693 ± 8402) than did the control mice (mean ± SE, 227,692 ± 33,642). The peritoneal macrophages were very efficient in terms of phagocytosis and showed a >1.5-fold higher activity than that of peripheral blood monocytes. We also tested the phagocytic activity of another control mouse strain (B10.A) and found that dysferlin-deficient SJL mice consistently showed significantly higher activity than did either of the normal control mouse strains (C57BL/6 or B10.A, data not shown).

To validate these data in human dysferlin-deficient patients, four patients were recruited into the Children's National Medical Center Clinical Research Center, and blood samples were drawn to evaluate monocyte phagocytic function. Four normal volunteers were studied at the same time. The untreated human monocytes showed a considerably lower level of phagocytosis of bacteria than did the mouse monocytes (Figure 2, A and B). Monocytes from LGMD2B patients showed a higher constitutive phagocytic activity than did normal control patients, but this difference was not statistically significant (Figure 2B).

We then tested the response of normal and dysferlin-deficient human monocytes to the proinflammatory cytokine TNF-α. Stimulation with TNF-α produced higher levels of response in LGMD2B patients than in normal patients (Figure 2B) and this increase is attributable to the TNF-induced decrease in phagocytic activity of normal monocytes. Further, when we treated SJL and normal C57BL/6 mouse monocytes with TNF-α, we found that TNF-α did not significantly affect the constitutively increased phagocytic activity of SJL/J monocytes (Figure 2A).

siRNA-Mediated Inhibition of Dysferlin Expression Significantly Enhances the Phagocytic Ability of the Mouse J774 Macrophage Cell Line

To verify that a loss of dysferlin protein expression is indeed responsible for the enhanced phagocytic activity of dysferlin-deficient monocytes, we designed siRNA oligonucleotides corresponding to four different regions of the dysferlin gene and transfected J774 cells with a cocktail of these siRNAs. Western blotting of cell lysates revealed a dose-dependent decrease in dysferlin expression in these cells after transfection with a range of concentrations of dysferlin siRNA, as compared to cells transfected with control siRNA at the same concentrations (Figure 3A). When we assessed the phagocytic activity of this macrophage-monocyte cell line after transient transfection with dysferlin and control siRNA oligos, we found that the dysferlin-specific siRNA oligos, but not the control oligos, significantly enhanced the phagocytic activity of the cells at 100 nmol/L (Figure 3B), in parallel with a significant loss of dysferlin expression at this concentration (Figure 3A). We also simultaneously tested the phagocytic activity of nontransfected J774 cells in the presence of

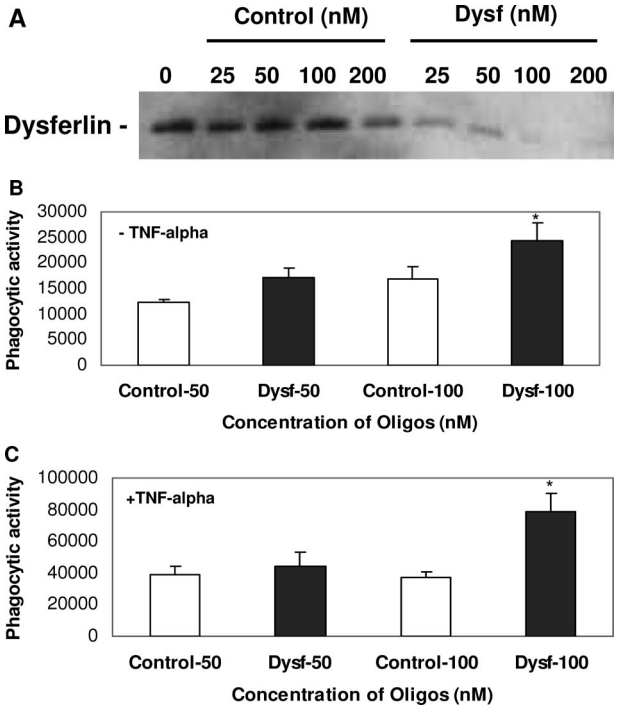

Figure 3. siRNA-mediated inhibition of dysferlin expression significantly enhances the phagocytic activity of the J774 macrophage cell line. **A:** J774 cells were transiently transfected with various concentrations of dysferlin for silencing or control siRNA. At 18 hours after transfection, cell lysates were prepared, and dysferlin protein was detected by Western blotting. **B** and **C:** Cells transfected with dysferlin siRNA show inhibition of dysferlin protein expression when compared to control siRNA-transfected cells. J774 cells were transiently transfected with 50 and 100 nmol/L of control and dysferlin siRNA oligonucleotides. The phagocytic activity in the absence (**B**) and presence (**C**) of 20 ng/ml of TNF-α was assessed, and the results are represented as phagocytic activity indices. Dysferlin-specific siRNA oligos significantly ($P < 0.05$) enhanced the phagocytic activity at 100 nmol/L, both in the presence (**C**) and absence (**B**) of TNF-α.

TNF-α stimulation and found that the phagocytic activity was twofold to threefold higher on TNF stimulation; transfection with dysferlin siRNA oligos in the presence of TNF-α also significantly enhanced phagocytosis (Figure 3C).

Characterization of Phagocytic/Endocytic Components in Human and Mouse Dysferlin-Deficient Muscle

Our data concerning dysferlin-deficient monocytes in both humans and mice suggested that phagocytic function is abnormal in these cells. Although muscle fibers are not considered phagocytic, they are likely to perform the related process of endocytosis, and dysferlin has been implicated in vesicular trafficking and repair.[3,4] For these reasons, we performed a candidate gene study of proteins with a role in endocytosis, using quantitative Western blot analysis of SJL and control mouse muscle. Western blotting demonstrated a clear loss of dysferlin in SJL muscle (Figure 4, A and B). We then analyzed the expression of several endocytic protein markers [cation-independent mannose 6 phosphate receptor (CIMPR), also called M6PR; adaptin-α; and clathrin]. All three markers of endocytosis showed significant up-regulation in Western blots of dysferlin-deficient muscle (Figure 4, A, and C–E).

At present the factors that initiate and perpetuate muscle inflammation are not well understood. We hypothesized that the enhanced phagocytic activity of dysferlin-deficient monocytes, coupled with alterations in the inflammatory regulators present at the muscle cell membrane, are able to initiate and perpetuate an inflammatory process in LGMD2B muscle. To test this hypothesis, we studied one such potential regulator of immune response, decay accelerating factor (DAF, or CD55). It is known that CD55 expression is dynamic and that this molecule is endocytosed and shed into the circulation.

DAF has been shown not only to suppress complement activation but also to inhibit T-lymphocyte and antigen-presenting cell responses.[33,34] Therefore, we examined DAF expression in a set of 128 human muscle biopsies from 13 diagnostic groups. DAF mRNA was found to be down-regulated in the dysferlin-deficient group when compared to normal controls ($P < 0.01$). However, this down-regulation was not dysferlin-specific because DAF mRNA was also reduced in HSP and FKRP patients (LGMD2I) (Figure 5D). To analyze the pattern of DAF expression in muscle, we stained the muscle biopsies of dysferlin-deficient and Duchenne muscular dystrophy patients and normal controls. In normal muscle, DAF expression was found in the capillaries and blood vessels and on muscle fibers. The normal muscle fibers showed an intense, uniform sarcolemmal expression (Figure 5A). In dysferlin patients, the expression on the surface of muscle fibers was patchy with vesicular pattern of internal reactivity (Figure 5B). Duchenne muscular dystrophy muscle fibers showed both continuous and discontinuous membrane staining patterns (Figure 5C). In both groups of patients, sarcolemmal staining varied significantly between and within the individual muscle fibers, ranging from continuous to almost no staining in a few fibers. Incubation with an isotype control or secondary antibody alone did not produce any staining (data not shown). These observations suggest that DAF may be one of several regulators that play a role in muscle inflammation.

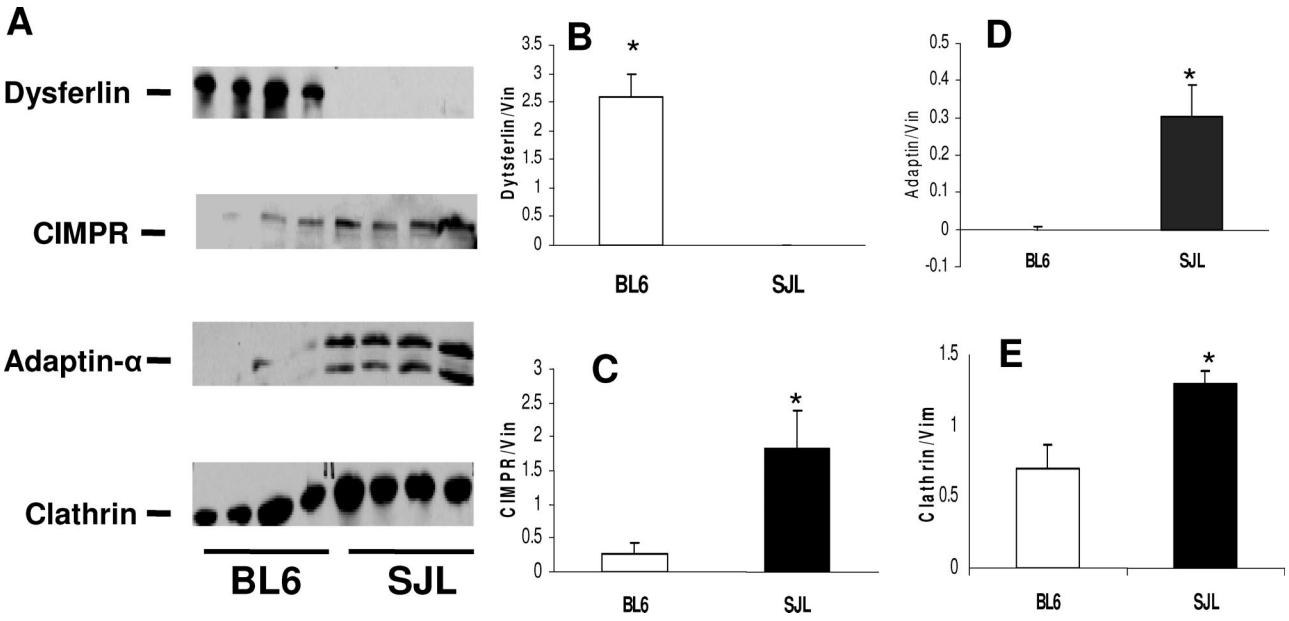

Figure 4. Dysferlin-deficient mouse muscle shows strong up-regulation of endocytotic proteins. **A:** Shown are Western blots from muscle lysates of C57BL/6J control and SJL/J (dysferlin-deficient) mice ($n = 4$ mice per group). As expected, the SJL/J muscle shows a dysferlin deficiency. Key proteins involved in endocytosis (CIMPR, adaptin-α, clathrin) are all strongly up-regulated in SJL/J muscle. **B–E:** All four blots were stripped and blotted for vinculin, and the ratios of the respective proteins and vinculin were calculated using Quantity One software and plotted as bar graphs: dysferlin (**B**), CIMPR (**C**), adaptin-α (**D**), clathrin (**E**).

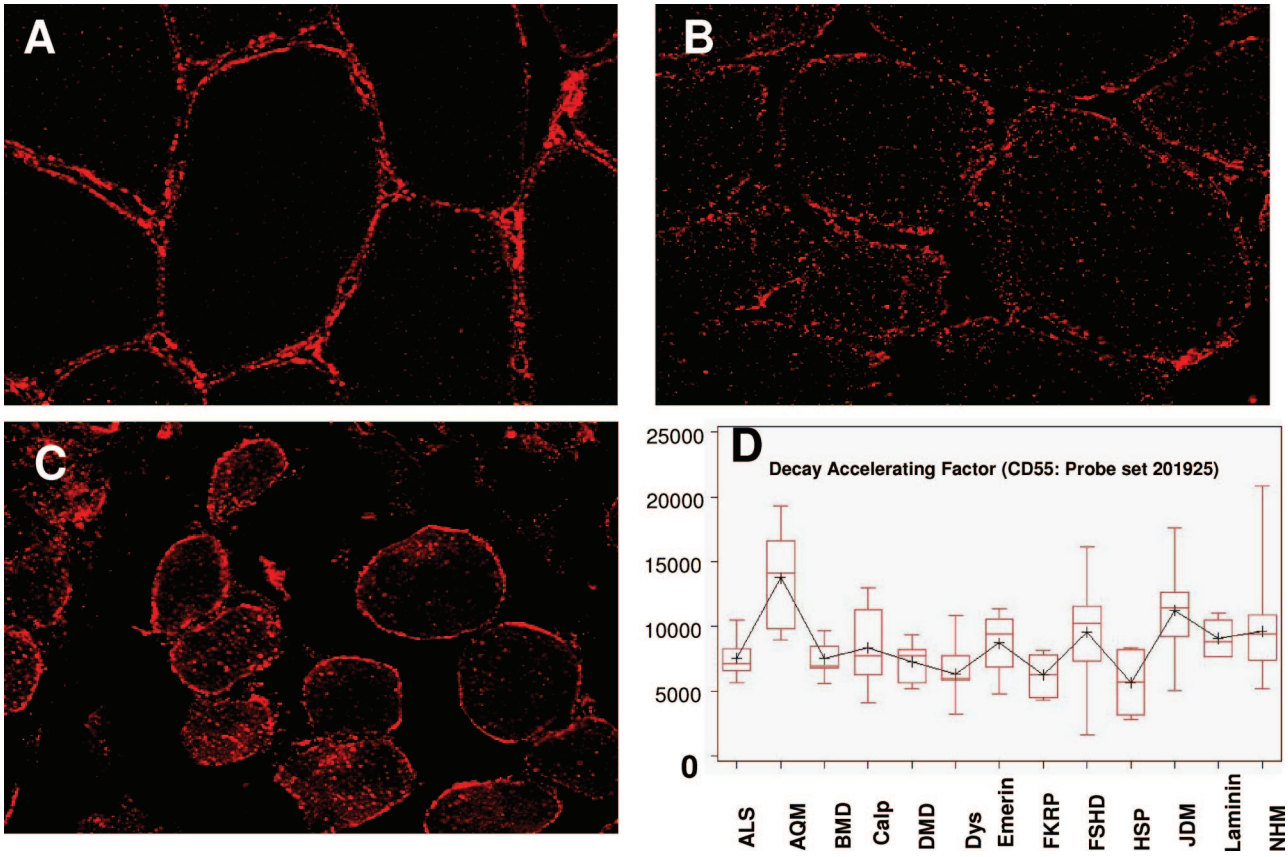

Figure 5. DAF is reduced in muscle biopsies from dysferlin-deficient patients. Frozen muscle biopsy sections of four normal (**A**), dysferlin-deficient (**B**), and Duchenne muscular dystrophy (**C**) patients each were stained with mouse anti-human DAF antibody and anti-mouse IgG Cy3. Representative sections from normal control and patient samples are shown. **A:** In normal muscle, DAF staining was uniformly present on muscle fibers (sarcolemma) as well as in blood vessels and capillaries. **B:** The DAF expression on muscle fibers of dysferlin patients was reduced and discontinuous. **C:** DAF expression varied significantly between muscle fibers in Duchenne muscular dystrophy patients; some fibers showed normal expression, and others showed low or no expression. **D:** Boxplots of DAF mRNA expression levels in 13 different neuromuscular diseases are shown. The total distribution is represented by six points. The box represents the middle 50% of the data (25th to 75th percentile), with the middle line representing the 50th percentile. The mean value is indicated by a +, and the minimum and maximum values are shown as the bottom and top hinges, respectively. The maximum, 75th percentile; median, 25th percentile, and minimum are represented by horizontal lines from the top down. Statistical significance of difference between dysferlin-deficient group and normal controls was performed by Student's *t*-test. Amyotrophic lateral sclerosis (ALS), acute quadriplegic myopathy (AQM), Becker muscular dystrophy (BDM), LGMD2A (Calp), Duchenne muscular dystrophy (DMD), LGMD2B (Dysf), Emery-Dreifuss muscular dystrophy (Emerin), fukutin-related protein deficiency (FKRP), fascioscapulohumeral dystrophy (FSHD), spastin haploinsufficiency (HSP), juvenile dermatomyositis (JDM), laminin A/C deficiency (laminin), and normal human muscle (NHM).

Expression of Rho Family Small GTPases Increases in Dysferlin-Deficient Splenocytes and Macrophages

It is known that Rho family small GTPase members Cdc42, Rac1, and RhoA regulate macrophage/dendritic cell adherence, motility, chemotaxis, endocytosis, and Ag presentation.[35] Therefore, we hypothesized that dysferlin deficiency leads to compensatory up-regulation of small Rho GTPases that control phagocytosis via actin cytoskeletal reorganization. The expression of Rac-1, RhoA, and Cdc42 is increased in splenocytes of dysferlin-deficient AJ mice (Figure 6, A and B). Because Cdc42 plays a critical role in phagocytotic and endocytotic processes we have further investigated its expression in peritoneal macrophages and muscle. Similar results were also observed in thioglycollate-induced peritoneal macrophages from dysferlin-deficient SJL mice (Figure 6C and unpublished data). Stimulation with lipopolysaccharide did not significantly affect the expression of Cdc42 in macrophages (Figure 6C). Further we also found a modest increase in the expression of Cdc42 in the skeletal muscle of normal and SJL mice (Figure 6D). These findings provide evidence that increase in phagocytosis seen in the absence of dysferlin is mediated because of the compensatory increase in the Rho family small GTPases.

Discussion

Dysferlin deficiency (LGMD2B) has an unusual histological and clinical presentation, in which strong and subacute inflammation of muscle is associated with disease onset, often leading to misdiagnosis of patients as having an inflammatory myopathy (polymyositis).[15,16] The emerging evidence showing that dysferlin plays important roles in vesicular trafficking in myofibers suggests that dysferlin could have a similar function in monocytes (and their derivative macrophages). In the present study, we have demonstrated the presence of activated mononuclear cells in muscle

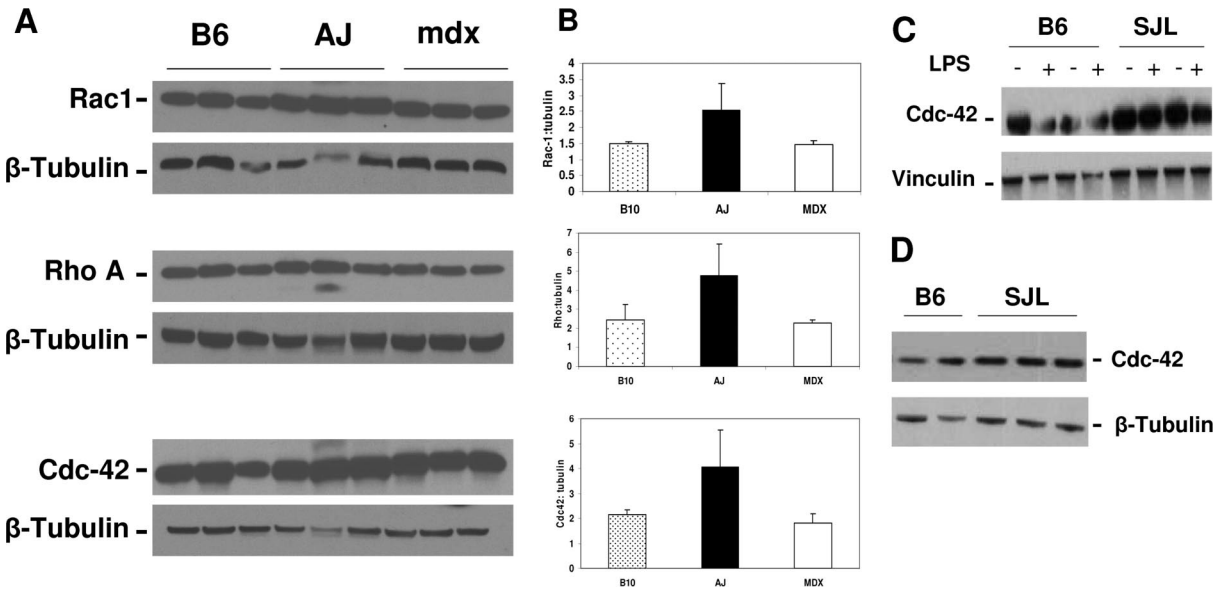

Figure 6. Expression of Rho family small GTPases in dysferlin-deficient cells. **A:** A representative Western blot of Rac-1, RhoA, and Cdc42 in the spleen lysates of dysferlin-deficient (AJ), normal (B6), and disease control (mdx) mice ($n = 3$ per mouse strain). **B:** Quantitation of levels of Rac-1, RhoA, and Cdc 42 relative to loading control β-tubulin in spleen lysates. **C:** A representative Western blot showing expression of Cdc42 is in dysferlin-deficient (SJL) thioglycollate-induced peritoneal macrophages. Lipopolysaccharide stimulation did not show significant difference in the CDC42 expression both in normal (B6) and dysferlin-deficient (SJL) mice. **D:** Cdc42 expression in dysferlin-deficient (SJL) and normal control (B6) muscle.

biopsies from dysferlin-deficient patients and mice. We have also found that both human and murine dysferlin-deficient monocytes show abnormally high phagocytic function. Whereas dysferlin-deficient mouse monocytes constitutively showed a significantly higher (~twofold) level of phagocytic activity than did normal control cells, human dysferlin-deficient monocytes showed an increase phagocytic activity over control cells at baseline but not at a level that was statistically significant. However, pretreatment of human dysferlin-deficient monocytes with TNF-α did cause a significant increase in phagocytic activity relative to normal controls, but a similar sensitivity to TNF-α was not seen in mice. These differences could potentially reflect an increased endogenous level of TNF-α in SJL/J mice, species differences, or the fact that the dysferlin-deficient macrophages had reached the upper threshold of their phagocytic ability. It is also possible that lack of significant response to TNF in human cells may be in part attributable to different mutations in different patients, differences in age, sex, and disease duration may. Taken together, our data suggest that phagocytosis of damaged myofibers in dysferlin-deficient LGMD2B patients may be inappropriately aggressive.

Our gene silencing experiments using dysferlin-specific siRNA have further defined the role of dysferlin in macrophage phagocytosis by clearly demonstrating that dysferlin siRNAs, but not control siRNAs, are able to enhance the phagocytic activity of the J774 macrophage cell line. Most importantly, our results indicate that the phagocytic defect seen in both human and murine monocytes is likely a direct consequence of the dysferlin deficiency, rather than a downstream effect on monocyte activation *in vivo* in the dystrophic organism. Experiments are currently in progress to define the role of dysferlin in macrophage endocytic and phagocytic processes.

To further explore the mechanism that leads to increase in phagocytosis in the absence of dysferlin, we have investigated the expression of Rho family small GTPases such as RhoA, Rac1, and Cdc42. These are signaling molecules involved in cytoskeleton remodeling and gene transcription and their activities are important not only for phagocytosis and endocytosis but also for myogenesis.[23,36] Efficient phagosomal maturation and elimination of ingested microbes in phagocytes is critically dependent on the activity of GTPases such as Cdc42.[37] Our findings strongly indicate that there is compensatory increase in the expression of Rho family small GTPases in dysferlin-deficient splenocytes, macrophages, and muscle contributing to the enhanced phagocytosis and endocytosis of innate immune cells.

Dysferlin-deficient murine myofibers have been shown to exhibit a loss of appropriate vesicular trafficking and membrane repair. However, our data on monocytes suggests an exaggerated endocytic response, rather than a loss of this response. In this context, it is important to note that we found a strong up-regulation of a number of endocytic protein markers (CIMPR, clathrin, adaptin-α) in both mouse and human dysferlin-deficient muscle. Murine dysferlin-deficient muscle has previously been shown to exhibit up-regulation of annexin A1 and A2, and dysferlin and annexins are binding partners.[11] Annexins A1 and A2 are known to promote the aggregation of intracellular vesicles and lipid rafts in a Ca^{2+}-dependent manner at the cytosolic plasma membrane in many cells.[38] In muscle membrane injury during normal or excessive use, a breach is thought to occur in the membrane, leading to an increase in the intracellular Ca^{2+}

concentration. This in turn results in an aggregation of intracellular vesicles to form a hydrophobic patch that is translocated and fuses with the sarcolemma to prevent further damage to the cell.[10] Dysferlin is proposed to act as a Ca^{2+}-dependent hook that contributes to the efficient fusion of the repaired area with the sarcolemma. Consistent with this model, vesicle accumulation has been observed adjacent to breaches in the plasma membrane in mouse muscle tissue from LGMD2B patient muscles. The up-regulation of vesicular trafficking proteins as well as Rho family small GTPases that we have described is consistent with an attempt to overcome the biochemical block posed by the dysferlin deficiency, through the compensatory up-regulation of proteins in the same biochemical pathway or alternative pathways.

In this context, it is important to address the underlying biochemical mechanisms that might be responsible for the increased phagocytic function that we observed in human and mouse monocytes. Macrophages show membrane remodeling as an early event during contact with cells at the site of target cell damage.[39] This membrane remodeling is thought to be preceded by transient changes in the concentration of calcium or other ions at the site of contact, leading to the initiation of membrane remodeling via Rho family small GTPases.[24,36,40] Thus, the initiating role of calcium in the repair of membrane damage in myofibers is likely similar to that of macrophage phagocytosis. Membrane repair in myofibers and phagocytosis in macrophages both clearly involve vesicle trafficking and many of the specific proteins commonly associated with this process. However, the end result is clearly a different process. Complicating prediction of molecular pathways is the compensatory up-regulation of pathway components in myofibers (shown here), and possibly macrophages. We hypothesize that the compensatory up-regulation of endocytotic proteins and Rho family small GTPases serves to rescue dysferlin-deficient myofibers, leading to a late-onset disease that is slowly progressive. A similar compensatory up-regulation in the context of decreased expression of negative regulatory molecules in muscle microenvironment could lead to enhanced immune activation and inflammation.

Recent studies have shown deposition of membrane attack complexes on nonnecrotic muscle fibers in dysferlin-deficient patients and have indicated that decreased expression of the complement regulator DAF on muscle fibers of dysferlin patients may increase their susceptibility to complement-mediated damage.[41,42] DAF also plays an important role in regulating T-cell and antigen-presenting cell responses, and its deficiency increases T-cell responses to antigen.[33,34,38] Therefore, our data suggest that decreased expression of this negative regulator of immune responses not only sensitizes muscle fibers to complement-mediated damage but may play a role in activating immune cells in the local microenvironment.

One of the primary functions of infiltrating tissue macrophages is to remove cellular debris (necrotic myofibers in the case of muscular dystrophy) through phagocytosis and digestion. Phagocytosis requires a complex and coordinated series of membrane reorganizations, in which the plasma membrane invaginates to form a pocket that is ultimately sealed to form a membrane-enclosed intracellular structure. This process is critically dependent on Rho family small GTPases such as RhoA, Rac1, and Cdc42. Because dysferlin has been shown to disrupt membrane trafficking in myofibers, we hypothesize that similarly disrupted membrane regulation in macrophages could interfere with the ability of macrophages to clear cellular debris appropriately.

A second important function of tissue macrophages is the coordination of cytokine signaling. Macrophages recognize secreted TNF-α, a potent proinflammatory cytokine, through TNF-α receptors located on the plasma membrane. This signaling is carefully regulated; when the TNF-α receptor binds extracellular TNF-α, it continues to transmit a proinflammatory signal into the cell until the extracellular domain of the receptor is cleaved by an adjacent protease.[43,44] Cleavage of the TNF-α receptor silences its signal; however, if there are disruptions in the regulation of the plasma membrane in dysferlin-deficient macrophages, this processing may be inhibited, resulting in TNF-α oversignaling. Thus, there may be ample opportunities for dysregulated macrophage/T-cell signaling if macrophage membrane or vesicle function is perturbed in dysferlin-deficient monocytes/macrophages.

SJL/J mice harbor at least two distinct gene mutations, one of which (*Dysf*^{im}) is in the dysferlin gene, where a 171-bp deletion causes a splice-site mutation, removal of 57 amino acids from the fourth C2 domain, and dramatic reductions in dysferlin protein.[45] The same strain also shows a mutation in a retinal degeneration gene (*Pde6b*^{rd1}) encoding a phosphodiesterase 6B protein in the rod cGMP receptors. The SJL/J mouse strain has long been used as a model for autoimmune disease. Although the relevant molecular pathways are not yet understood, SJL/J mice are known to be sensitized to the development of autoimmune disease, with regard to encephalitis,[46,47] myositis (immunization with myosin),[48–50] and colitis (immunization with collagen).[46,51] These mice are also known to exhibit subclinical myopathy; the discovery that they have a mutation in the dysferlin gene provided the explanation for this myopathy, but the heightened propensity of these mice to develop autoimmune disease remains very poorly understood.

Our data are consistent with overactive macrophage activity in LGMD2B patients, which would be expected to lead to an exaggerated injury response. Because dysferlin-deficient myofibers are continuously damaged by faulty membrane repair, we hypothesize that the loss of negative regulators of the immune response such as DAF along with increase in Rho family small GTPases in immune cells may enhance the immune and inflammatory cell activity in some dystrophies. This enhancement could lead to an overaggressive clearance of damaged myofibers and/or excessive signaling to immune cells, producing an exaggerated and damaging immune cell response. Consistent with this model, macrophage signaling is increasingly recognized as critical to mediating immune responses and to promoting tissue regeneration.[52]

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