

## Research Article

# Distinct Effects of Contraction-Induced Injury *In Vivo* on Four Different Murine Models of Dysferlinopathy

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Mutations in the *DYSF* gene, encoding dysferlin, cause muscular dystrophies in man. We compared 4 dysferlinopathic mouse strains: SJL/J and B10.SJL-*Dysf*<sup>flm</sup>/AwaJ (B10.SJL), and A/J and B6.A-*Dysf*<sup>prmd</sup>/GeneJ (B6.A/J). The former but not the latter two are overtly myopathic and weaker at 3 months of age. Following repetitive large-strain injury (LSI) caused by lengthening contractions, all except B6.A/J showed ~40% loss in contractile torque. Three days later, torque in SJL/J, B10.SJL and controls, but not A/J, recovered nearly completely. B6.A/J showed ~30% torque loss post-LSI and more variable recovery. Pre-injury, all dysferlinopathic strains had more centrally nucleated fibers (CNFs) and all but A/J showed more inflammation than controls. At D3, all dysferlinopathic strains showed increased necrosis and inflammation, but not more CNFs; controls were unchanged. Dystrophin-null *DMD*<sup>mdx</sup> mice showed more necrosis and inflammation than all dysferlin-nulls. Torque loss and inflammation on D3 across all strains were linearly related to necrosis. Our results suggest that (1) dysferlin is not required for functional recovery 3 days after LSI; (2) B6.A/J mice recover from LSI erratically; (3) SJL/J and B10.SJL muscles recover rapidly, perhaps due to ongoing myopathy; (4) although they recover function to different levels, all 4 dysferlinopathic strains show increased inflammation and necrosis 3 days after LSI.

## 1. Introduction

The *DYSF* gene encodes dysferlin, a tail-anchored integral membrane protein of 230 kDa that is thought to mediate membrane stability or fusion through the action of calcium-binding C2 domains in its large cytoplasmic region [1–3]. Mutations in dysferlin have been linked to several muscular dystrophies in man, including limb girdle muscular dystrophy type 2B (LGMD2B) and Miyoshi myopathy (MM) [4, 5]. There are currently no treatments for these diseases, but several therapeutic approaches have been tested *in vitro* and in mice, including gene therapeutic studies. The success of these experiments and their ultimate translation to the clinic will rely on the availability of murine models of the human dysferlinopathies. Several such models exist, including two that, like the human dystrophies, arose spontaneously and two that were generated by targeted gene ablation [6, 7]. Although some of the properties of these strains of dysferlinopathic mice have been described, very few of their

characteristics have been directly compared to determine which, if any, most closely reproduce the characteristics of LGMD2B/MM. Here, we report the results of comparative studies on the two spontaneously occurring murine dysferlin mutants, SJL/J and A/J, and of the two strains derived from these mutants by breeding them into C57Bl mice, B10.SJL-*Dysf*<sup>flm</sup>/AwaJ and B6.A-*Dysf*<sup>prmd</sup>/GeneJ (referred to in this paper as B10.SJL and B6.A/J, resp.).

SJL/J mice show spontaneous myopathy [7–9], due to a mutation in the *Dysf* gene that results in the deletion of an exon encoding 57 amino acids in one of dysferlin's C2 domains [10, 11]. This mutation leads to a ~6-fold or greater reduction in dysferlin levels in SJL/J muscle [7, 12] and is associated with the development of fiber necrosis, central nucleation, inflammation, elevation of serum creatine kinase, and, in some muscles, fatty infiltration [13]. Some phenotypic changes occur in mice as early as 3 weeks of age and additional changes, including increases in centrally nucleated fibers (CNFs), are evident in 2-month-old mice

[9, 10, 12, 13]. The myopathy becomes more prominent with age, with a measurable loss of strength at 6 months of age and even greater compromise of histopathology and function in older animals [9, 13]. However, when compared to control strains, SJL/J mice also show behavioral differences, a high incidence of reticulum cell sarcomas [14], elevated levels of circulating T cells, and greater susceptibility to autoimmune diseases (<http://jaxmice.jax.org/strain/000686.html>). These traits are probably not linked to its dystrophic phenotype, which has been bred into the C57Bl/10J background [10, 12]. Extensive comparisons of SJL/J mice with their B10.SJL counterparts have not been reported, but preliminary studies of the time course of development of overt myopathy and the nature of the myopathy in these strains seem similar [9, 12]. They also share the property that proximal muscles, such as the quadriceps femoris, are more severely affected than distal muscles, such as the gastrocnemius and tibialis anterior [9, 12].

A/J, the other spontaneous dysferlinopathic mouse, has an ETn retrotransposon insertion in intron 4 of the murine *Dysf* gene that leads to the complete absence of dysferlin as assayed by immunoblots [7]. Paradoxically, the A/J mouse shows a milder phenotype at young ages and only becomes overtly myopathic at ages approaching 1 yr [7]. Nevertheless, serum creatine kinase is as elevated in 1-month-old A/J mice as it is in SJL/J mice [7]. As also seen in SJL/J and B10.SJL mice (see above), the effect of the A/J mutation is more pronounced in proximal than in distal muscles [7]. Ultrastructural images of A/J muscle, like those of SJL/J and dysferlin-null mice and human dysferlinopathies [6, 7, 15], show lesions in the sarcolemma and the accumulation of membrane-bound vesicles nearby as well as changes in the basal lamina [7]. Comparisons of A/J mice with mice carrying the same retrotransposon element in the C57Bl/6J background, B6.A/J, suggest that the hindlimb muscles of the latter develop myopathy more rapidly than A/J mice [16–18]. A/J mice carry a number of other mutations, however, that lead to hearing loss, a high incidence of lung carcinomas, lack of complement 5, and resistance to diabetes (<http://jaxmice.jax.org/strain/000646.html>). They also show much lower levels of spontaneous motor activity than many other strains of mice. These characteristics, or others, may modulate the extent and development of dysferlinopathy in A/J mice.

Our earlier studies, which focused on the A/J strain, showed that eccentric injuries caused by repetitive large-strain lengthening contractions had distinct effects on the ankle dorsiflexor muscles of A/J mice, compared to several control strains. In particular, both the A/J and control mice were equally susceptible to the large-strain injury, losing 40–50% of the contractile torque of their ankle dorsiflexors. Immediately after injury and for several days thereafter, the myofibers of A/J mice retained 10 kDa fluorescein-dextran, to the same extent as controls, suggesting that they had resealed their injured sarcolemmal membranes as well as controls. We interpreted these results to mean that, contrary to the conclusions reached by earlier studies performed on isolated myofibers *in vitro* [6], dysferlin is not needed for repairing sarcolemma damaged by a physiological injury *in vivo*. Differences between controls and A/J appeared

subsequent to injury, however, as controls recovered within 3 days of the injury, with little necrosis or inflammation and without requiring myogenesis, whereas the A/J mice required several weeks to recover, due to extensive fiber loss following necrosis and massive inflammation and subsequent regeneration via myogenesis.

Here we compare the responses of the four strains of mice, A/J, B6.A/J, SJL/J, and B10.SJL, to injury caused by large-strain lengthening contractions. In particular, we compare their baseline torque generated by the ankle dorsiflexor group, the susceptibility to injury of this muscle group to repetitive large-strain lengthening contractions, the recovery of torque 3 days after injury, and the histological status of the muscles before and after injury, including assessments of central nucleation, necrosis and inflammation. Our results suggest that the B6.A/J, SJL/J, and B10.SJL all recover from the injury within 3 days following injury, whereas A/J do not. Nevertheless, the response of the B6.A/J is distinct from that of SJL/J and B10.SJL. We also compare our findings on dysferlinopathic mice with dystrophin-deficient C57Bl/10ScSn-Dmd<sup>mdx</sup>/J mice (DMD<sup>mdx</sup>). We conclude that the dysferlinopathic strains of mice examined in this study are useful for studying different stages in the development of dysferlinopathy and the altered response to contraction-induced injury in the absence of dysferlin.

## 2. Materials and Methods

We studied baseline contractile torque, change in torque following large-strain lengthening contractions, and histological changes 3 days after injury in 4 different murine models of dysferlin deficiency and compared them to their respective control strains and a murine model of dystrophin deficiency.

All mice used in this study were obtained either directly from the Jackson Laboratory (Bar Harbor, ME) or were obtained from our animal colonies set up from breeding pairs initially obtained from the Jackson Laboratory (see Table 1). Mice used for this study were males between 12–16 wks of age. All experimental protocols involving animals were approved by the Institutional Animal Care and Use Committee of the University of Maryland School of Medicine, Baltimore.

**2.1. Torque Measurement and Large-Strain Injury (LSI).** Measurements of contractile torque and large-strain injury were performed *in vivo* as described [17–20] with the Small-animal Unit for Muscle Injury, Muscle Testing and Muscle Training (SUMITT, patent pending). Briefly, an anesthetized animal was placed on its back with its foot strapped onto a footplate in series with a torque transducer. The tibia was stabilized with a pin. The dorsiflexor muscles were stimulated via the peroneal nerve with a bipolar transcutaneous electrode. Twitches elicited by single 0.1 ms pulses were used to optimize electrode placement, current amplitude, and the optimal tibiotarsal angle for the dorsiflexors (typically 110° of plantarflexion). Post-LSI torque was measured at the same preinjury testing angle. In this study we did not test if LSI alters the torque-angle relationship of the dorsiflexors although our unpublished data suggest no change in

TABLE 1: Mouse strains. Mouse strains used in the study and number of animals studied per strain.

Strain	Source	JAX mice database stock #	DYSF mutation	N
A/WySnJ	School of Medicine University of Maryland. Breeding pairs from The Jackson Laboratory	000647	N/A. Control strain for A/J mice	8
A/J	School of Medicine University of Maryland. Breeding pairs from The Jackson Laboratory	000646	A retrotransposon insertion within intron 4, causing aberrant splicing of the gene	8
C57Bl/6J	The Jackson Laboratory	000664	N/A. Background strain for B6.A/J	8
B6.A/J	School of Medicine University of Maryland. Breeding pairs from The Jackson Laboratory	012767 B6.A-Dysf <sup>prmd</sup> /GeneJ	Progressive muscular dystrophy (prmd) allele from A/J introgressed into the C57BL/6J genetic background	8
SWR/J	The Jackson Laboratory	000689	N/A. Control strain for SJL/J mice	8
SJL/J	The Jackson Laboratory	000686	171 bp in-frame deletion in the encoded mRNA is predicted to remove 57 amino acids from dysferlin	8
C57BL/10J	The Jackson Laboratory	000665	Background strain for B10.SJL	8
B10.SJL	School of Medicine University of Maryland. Breeding pairs from The Jackson Laboratory, and Dr. A. J. Wagers, Harvard University, Cambridge, MA.	011128 B10.SJL-Dysf <sup>im</sup> /AwaJ	DYSF mutation (inflammatory myopathy, im) in the SJL strain introgressed into the C57BL/10ScSnHim strain and subsequently bred to C57BL/10J	8
DMD <sup>mdx</sup>	School of Medicine University of Maryland. Breeding pairs from The Jackson Laboratory	001801 C57BL/10ScSn-Dmd <sup>mdx</sup> /J	N/A. Dystrophin-deficient mice. C-to-T transition at position 3185, resulting in a termination codon in place of a glutamine codon	8

A/WySnJ and A/J after LSI. Increasing frequencies of pulses within a 300 ms pulse train were used to determine optimal tetanic frequency, which was 80 or 90 Hz for all control strains and A/J and 100–125 Hz for all other strains. After optimal baseline torque (maximal twitch and tetany) was recorded, 20 lengthening contractions were induced in the dorsiflexors by moving the foot into plantarflexion from 90–180° (starting with foot orthogonal to tibia) at 1200°/s, while the dorsiflexors were tetanically stimulated. While the 180° of plantarflexion is unlikely to occur in mice during normal motor activity, our injury model is physiological as it does not exceed the maximal range of plantarflexion of the ankle-foot complex recorded through gentle passive movement. A rest period of 1 min was allowed between successive lengthening contractions. Torques after LSI were recorded 4 min after the last lengthening contraction. Contractile data in Figures 1 and 2 are from 6 animals that were studied longitudinally on the day of injury (D0) and 3 days later (D3). We performed a terminal study on two animals of each strain at D0 to analyze histological changes immediately following injury (middle panels in Figure 3; contractile data from these animals are not shown).

**2.2. Histological Studies.** Histological studies were performed on the *Tibialis anterior* (TA) muscle, as it accounts for nearly

90% of the force produced by the dorsiflexors in mice [21]. Control and injured muscles were harvested from 2 animals per strain ~10 min after injury on D0 and from 6 animals per strain at D3 for histological studies. Collection of unfixed tissue, hematoxylin and eosin (H & E) staining, and labeling of macrophages with rat anti-mouse antibodies to CD68, which labels all murine macrophages [22], were performed as described [18, 23]. Quantitative data from H & E staining are from 8–10 unique visual fields (20x objective) from 3 different animals and expressed as a percentage of the total number of myofibers counted (~500 fibers counted for each strain of mice). Macrophage counts are from 3 animals per strain from 4 unique visual fields (40x objective) per muscle and expressed as the number of cells per square mm.

**2.3. Statistical Methods.** All statistical analyses were performed with SigmaStat (Ashburn, VA). Baseline torque data were analyzed with a one-way ANOVA, injury and recovery data were analyzed with a repeated measures two-way ANOVA. CNFs, necrotic fibers and macrophage counts (CD68+ cells) were analyzed by two-way ANOVAs. *Post hoc* analyses were performed according to the Student-Newman-Keuls method. Linear regression analyses of the effect of necrosis and macrophage counts torque as well the effect of macrophage counts on necrosis were performed with

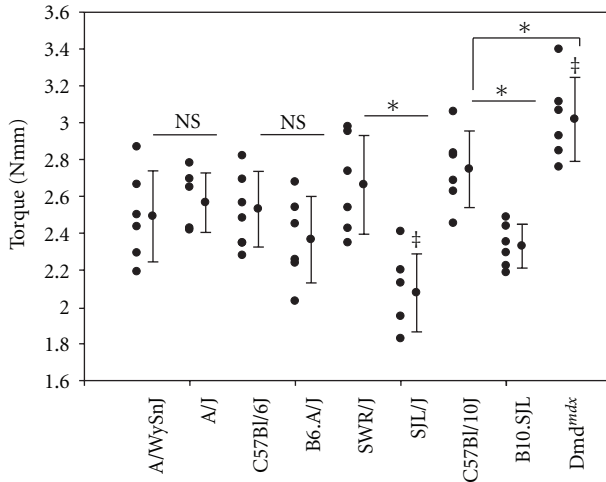


FIGURE 1: Baseline torque before large-strain injury. Maximum isometric tetanic torque of the dorsiflexor muscle group was measured prior to LSI. All control strains (A/WySnJ, C57Bl/6J, SWR/J, and C57Bl10/J) produced a similar level of contractile torque. Among the *Dysf* mutants, only SJL/J mice and B10.SJL mice were weaker than their corresponding controls. SJL/J mice were also weaker than A/J mice. Raw torque produced by DMD<sup>mdx</sup> was the highest among the studied strains of mice. \*Significant difference compared to control strain at same time point. NS:no significant difference. †Significant difference compared to A/J at same time point.

SigmaStat. Alpha was set at 0.05 *a priori*, and *P* values less than 0.05 were considered significant. Physiological data are reported as mean  $\pm$  S.D., and histological data are reported as mean  $\pm$  S.E.M.

### 3. Results

We used established physiological and morphological methods to compare 4 strains of dysferlin-deficient mice, A/J and SJL/J, which carry different mutations of dysferlin, and B6.A/J and B10.SJL, which carry their respective mutations in the C57Bl/6J and C57Bl/10J backgrounds. We used the latter as controls as well as SWR/J and A/WySnJ mice as controls in our studies of SJL/J and A/J mice, respectively (see Table 1). Our physiological experiments measured the contractile torque generated by the ankle dorsiflexor muscles, the loss of torque following a large-strain injury caused by repeated lengthening contractions, and the recovery of torque 3 days after injury. Our histological experiments measured the number of CNFs, necrotic myofibers, and inflammatory macrophages in TA muscles before and after injury. Our results indicate that these 4 strains of dysferlinopathic mice share some important features but differ significantly in others.

**3.1. Physiological Assays.** We first measured the torque generated by the ankle dorsiflexor muscles of the 4 types of dysferlinopathic mice, compared to the appropriate control strains (Figure 1). All control strains were statistically indistinguishable from each other. There was no statistical difference between A/J mice and its control, A/WySnJ mice,

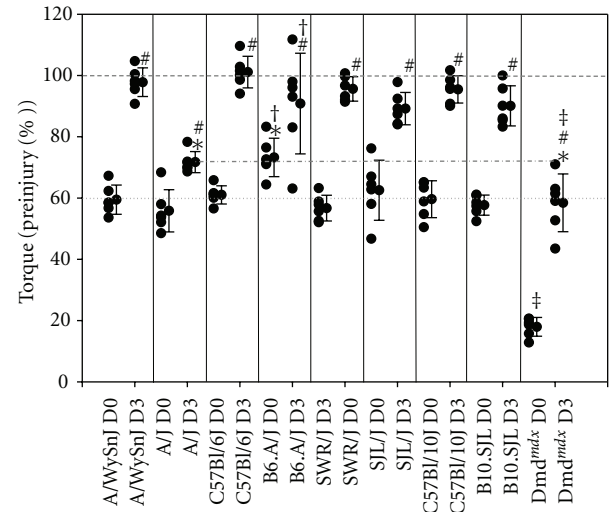


FIGURE 2: Torque after LSI. We measured maximum isometric torque  $\sim$ 4 min after the last lengthening contraction in the LSI protocol (D0) and again 3 days later (D3). All control strains lost a similar level of torque. All dysferlin mutant mice except B6.A/J showed a similar reduction in torque immediately after injury; B6.A/J mice showed less susceptibility to initial injury compared to control C57Bl/6J mice as well as other dysferlinopathic strains. DMD<sup>mdx</sup> mice showed the highest level of torque loss immediately after injury. At D3, all control strains recovered to near baseline torque levels. All dysferlin mutants except A/J recovered to similar levels as their respective control strains. A/J mice and DMD<sup>mdx</sup> mice showed a significant torque deficit at D3. \*Significant difference compared to control strain at same time point. #Significant difference within same strain between D0 and D3. †Significant difference compared to same *Dysf* mutation at same time point. ‡Significant difference compared to A/J at same time point.

and between B6.A/J and its control, C57Bl/6J. SJL/J and B10.SJL mice generated similar levels of torque, but were weaker than their respective control strains, SWR/J and C57Bl/10J. All dysferlinopathic strains and their controls were significantly weaker than DMD<sup>mdx</sup> mice, consistent with earlier reports that the latter are stronger than controls [24–26]. When normalized to muscle weight, however (see Table 2), TA muscles of DMD<sup>mdx</sup> mice were  $\sim$ 38% weaker than muscles of C57Bl/10J mice.

We next subjected each murine strain to our large-strain injury protocol (LSI, see Methods). All strains except B6.A/J lost  $\sim$ 40% of their preinjury torque after LSI (Figure 2). B6.A/J mice only lost  $\sim$ 30% of their baseline torque, which was significantly less than its control C57Bl/6J and all other strains (Figure 2; supplementary data, Figures S1 and S2 available online at doi:10.1155/2011/134031). In contrast to all the control and dysferlinopathic strains we assayed, DMD<sup>mdx</sup> mice showed a  $\sim$ 80% loss of contractile torque after LSI, significantly greater than any of the other strains, consistent with the greater susceptibility to injury by lengthening contractions characteristic of these mice [27–29].

We assayed the ability of mice of each strain to recover from the large-strain injury within the 3-day period that we previously found was sufficient for controls [17–19].



TABLE 2: Weights of TA muscles. Wet weights (in mg) of uninjured (right) and injured (left) TA muscles collected at D3 are given.

	A/Wy SnJ	A/J	C57Bl/6J	B6.A/J	SWR/J	SJL/J	C57Bl/10J	B10.SJL	DMD <sup>mdx</sup>
<i>N</i>	4	4	4	4	3	3	3	3	3
D3 control	43 ± 1	42 ± 1	51 ± 1	48 ± 1	45 ± 4	50 ± 2	43 ± 2	45 ± 1	79 ± 2
D3 injured	45 ± 3	40 ± 1	52 ± 1	47 ± 2	47 ± 5	48 ± 4	45 ± 2	46 ± 1	75 ± 4

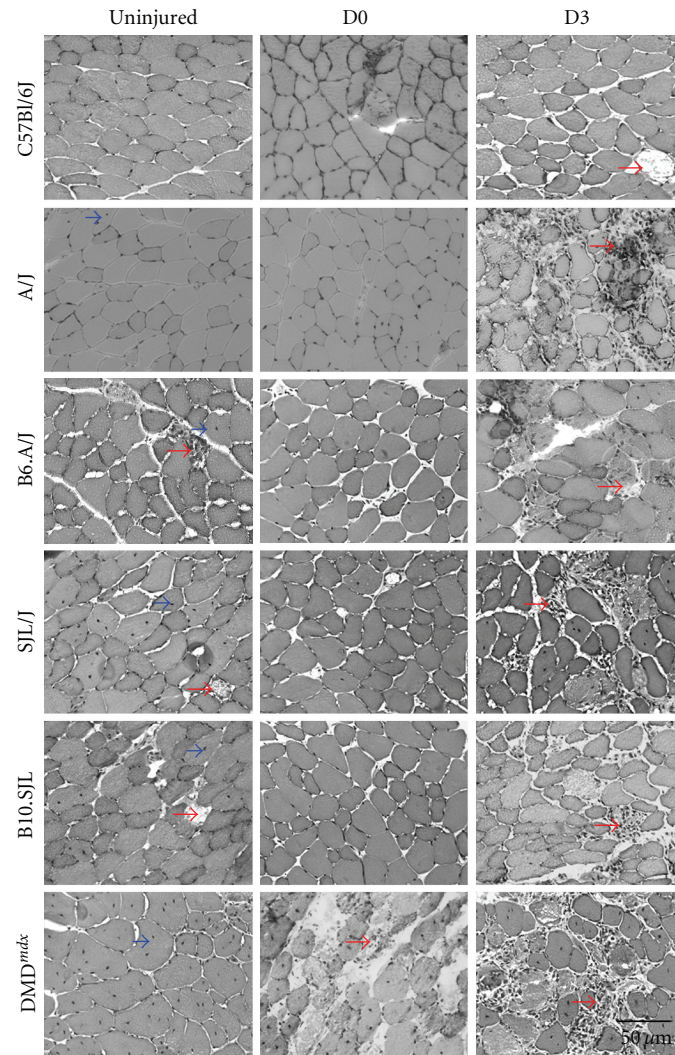


FIGURE 3: Histology before and after injury. We labeled TA muscles with H&E stains. In uninjured muscle, necrotic fibers identified by pale, disrupted cytoplasmic staining and infiltrated by mononuclear cells appeared in all dysferlinopathic strains except A/J (red arrows). All dysferlin mutants had many CNFs (blue arrow heads) before LSI. At D3 post-LSI, necrotic fibers increased in all mutants, with the most widespread area of necrosis in A/J muscle. Extensive myofiber damage soon after LSI was visible in DMD<sup>mdx</sup> muscle, but not in control or dysferlinopathic muscle.

The mean torque for C57Bl/6J mice exceeded 100% baseline torque, suggesting complete recovery of function 3 days after LSI. All other control strains were statistically indistinguishable from C57Bl/6J at D3 post-LSI, indicating that they too had regained all of their lost contractile torque (Figure 2). As reported [17–19], A/J mice only recovered to only ~70% of their preinjury torque, equivalent to ~1/4 of the torque lost immediately after LSI. Surprisingly, B10.SJL and SJL/J recovered to ~90% of their original torque by D3 (Figure 2), which was also statistically indistinguishable from the level

of recovery shown by their respective control strains. This suggests that the absence or severe depletion of dysferlin is not sufficient to delay the recovery of torque 3 days after large-strain injury. The group mean for contractile torque for B6.A/J also recovered as well as their control C57Bl/6J mice. However, their recovery at D3 showed a wide range of values from a loss of 8% below initial injury in one animal to a gain of 10% above preinjury torque in another. Due to the high level of variability seen in the set of 6 B6.A/J animals, we performed physiological measurements on additional

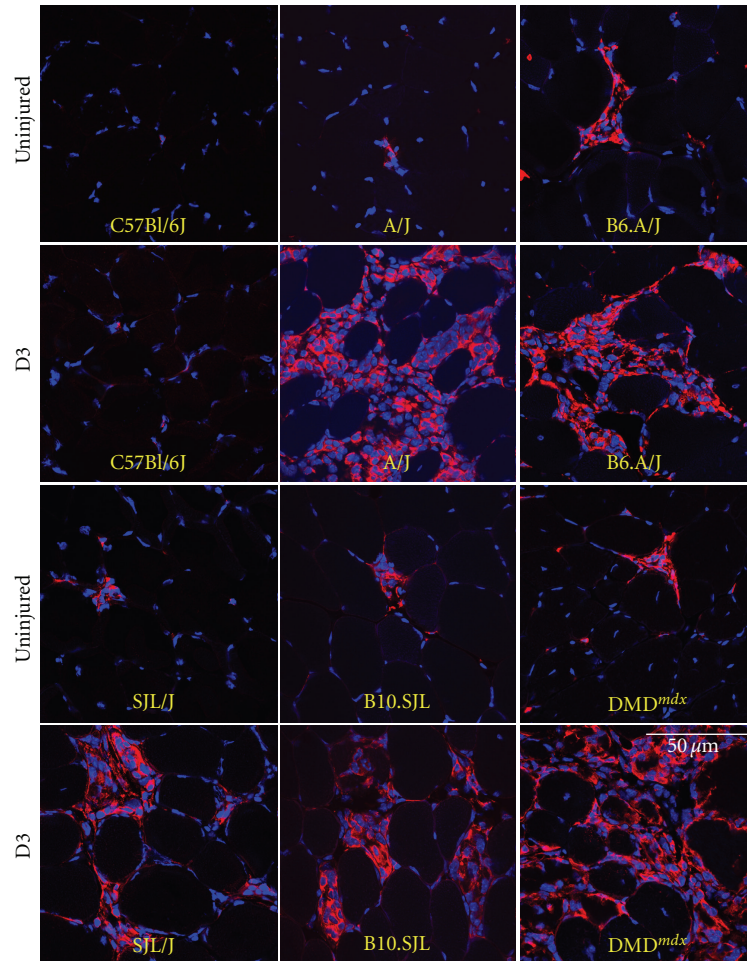


FIGURE 4: Macrophages (CD68+ cells) in muscle before and after injury. We labeled cross sections of uninjured and injured TA muscles with antibodies to CD68 to identify macrophages (red) and DAPI to mark nuclei (blue). Focal areas of macrophage infiltration were evident in uninjured muscle of all dysferlinopathic strains as well as  $DMD^{mdx}$  mice. At D3 following LSI, macrophage numbers increased significantly in these mutant strains.

sets of animals. With increased sample size, B6.A/J were statistically indistinguishable from C57Bl/6J and A/J at D0, but were different from both these strains at D3. They were also more variable (supplementary data, Figures S1 and S2). Just as they differed in their initial torque and susceptibility to injury,  $DMD^{mdx}$  mice also differed significantly from the control and dysferlinopathic strains in their extent of recovery from LSI, reaching only ~60% of their original torque by D3, equivalent to ~1/2 of the torque lost initially.

**3.2. Histological Studies.** We assessed 3 different morphological markers of myopathy, CNFs and necrotic fibers (both quantitated from H&E-stained sections) and macrophages (quantitated from sections immunolabeled with antibodies to CD68), in uninjured TA muscles and in muscles 3 days after injury (Figures 3 and 4). As all control strains were statistically indistinguishable at D0 and D3 in the number of CNFs, necrotic fibers, and CD68+ cells, we only show comparisons for these variables with C57Bl/6J data.

In uninjured muscles, CNFs were elevated in all dysferlinopathic mice as well as in  $DMD^{mdx}$  (Figure 5), compared

to controls. Uninjured SJL/J muscle had more CNFs than the other dysferlinopathic strains. We found no significant differences in central nucleation among B10.SJL, A/J, and B6.A/J.  $DMD^{mdx}$  mice showed much higher levels of CNFs, consistent with earlier reports [30, 31]. Except for  $DMD^{mdx}$ , we observed no significant increases in CNFs at D3 in any of the strains.

Although necrotic fibers were readily detected in the uninjured TA muscles of B6.A/J, B10.SJL, and SJL/J mice, the numbers were not significantly greater than control muscles (Figures 3 and 6). On D3 post-LSI, however, the number of necrotic fibers increased significantly in all of the dysferlinopathic mice (Figure 6). Necrotic fibers at D3 in A/J and SJL/J muscles were statistically indistinguishable, but B6.A/J and B10.SJL muscles had significantly fewer necrotic fibers than A/J and SJL/J, respectively.  $DMD^{mdx}$  had the highest levels of necrosis, which can be explained by severe myofiber damage seen immediately after injury, a feature not seen in dysferlinopathic muscle (Figure 6; also see middle panels in Figure 3). A regression analysis with % necrotic fibers as the independent variable and torque on D3

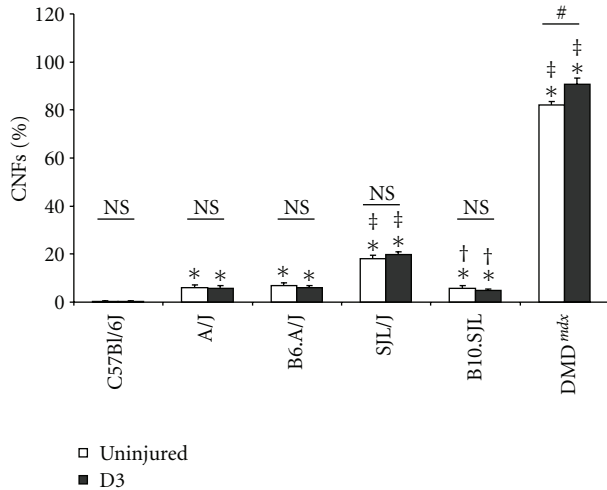


FIGURE 5: Quantitation of CNFs. We counted CNFs in H&E-stained cross sections of uninjured and injured TA muscles. In uninjured muscles, CNFs were elevated in all dysferlin mutants, with SJL/J muscle showing the greatest elevation. DMD<sup>mdx</sup> muscle showed several-fold CNFs more than all the dysferlinopathic samples. Except for DMD<sup>mdx</sup>, none of the strains showed changes in CNFs at D3. \*Significant difference compared to control strain at same time point. #Significant difference within same strain between uninjured and D3. †Significant difference compared to same *Dysf* mutation at same time point. ‡Significant difference compared to A/J at same time point. NS:no significant difference.

as the dependent variable showed a linear relationship (Figure 7; adjusted  $r_2 = 0.904$ ,  $P = 0.002$ ). Thus, the deficit in torque 3 days after LSI was directly proportional to the number of necrotic myofibers at D3. SJL/J alone fell above the 95% confidence interval suggesting that their D3 contractile torque exceeds what would be expected based on the level of necrosis. Consistent with this, SJL/J mice have been shown to recover from other forms of injury very efficiently [32].

We also quantitated the number of macrophages (CD68+ cells) in TA muscles of each strain, both before and after injury. Focal areas of necrosis in uninjured muscles were invaded by macrophages in all dysferlinopathic strains (Figure 4). However, macrophage counts were not significantly different from uninjured C57Bl/6j (Figure 8). All dysferlinopathic strains showed significant increases in macrophages at D3 post-LSI (Figure 8), with the highest counts detected in A/J. DMD<sup>mdx</sup> muscle at D3 had significantly greater macrophage infiltration than A/J. Regression analysis with D3 torque regressed on macrophage counts showed a linear relationship (Figure 9(a); adjusted  $r_2 = 0.836$ ,  $P = 0.007$ ), with all strains falling within the 95% confidence interval. Similarly, a regression analysis with macrophage counts regressed on necrosis at D3 also showed a linear relationship (Figure 9(b); adjusted  $r_2 = 0.896$ ,  $P = 0.003$ ), with all strains falling within the 95% confidence interval.

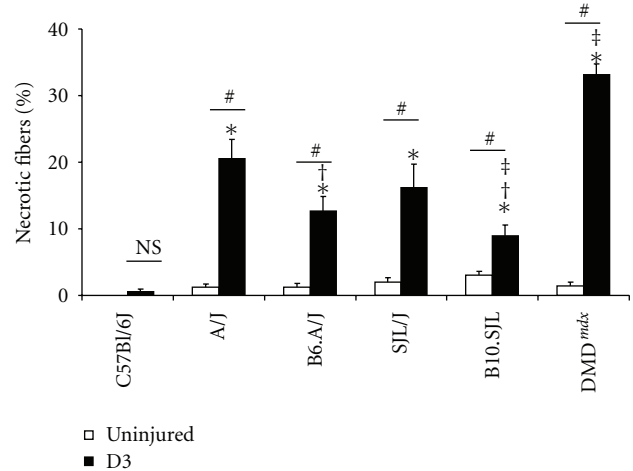


FIGURE 6: Quantitation of necrotic fibers. We counted necrotic fibers in H&E-stained cross sections of uninjured and injured TA muscles. Necrotic fibers increased significantly at D3 in all mutant strains. A/J muscle showed the largest increase among the dysferlin mutants, but the extent of necrosis in A/J muscles was significantly less than DMD<sup>mdx</sup> at this time point. \*Significant difference compared to control strain at same time point. #Significant difference within same strain between uninjured and D3. †Significant difference compared to same *Dysf* mutation at same time point. ‡Significant difference compared to A/J at same time point.

#### 4. Discussion

Several strains of dysferlinopathic mice have been studied as possible models of human muscular dystrophies linked to mutations in the dysferlin gene. These include 2 different strains generated by gene ablation [6, 7], 2 strains with spontaneous mutations in the dysferlin gene, A/J and SJL/J, and the strains that were derived by breeding the A/J and SJL/J *Dysf* mutations into the well-established C57Bl/10J and C57Bl/6j genetic backgrounds, namely, B6.A/J and B10.SJL. Although several laboratories have compared some of these strains to each other, with results suggesting that they share similar properties [7, 12, 13], few direct comparisons have been made of mice carrying the SJL/J mutation to those carrying the ETn retrotransposon of A/J, and none have focused on their responses to injuries caused by lengthening contractions. Here we provide evidence to suggest that SJL/J and B10.SJL have a similar phenotype. We show further that they differ significantly from A/J and B6.A/J mice and, moreover, that the latter two strains differ significantly from each other.

Our LSI protocol, which differs from many other methods for injuring the hindlimb muscles of mice, has several advantages, including rapidity and reproducibility. Our LSI model also clearly distinguishes between some dysferlinopathic strains and controls, including A/J and A/WySnJ. Furthermore, the LSI model also distinguishes between some of the dysferlinopathic mice we have examined, discussed further below, as well as between dysferlin-deficient and dystrophin-deficient strains. LSI causes ~2-fold greater force deficit immediately after LSI in muscles lacking dystrophin



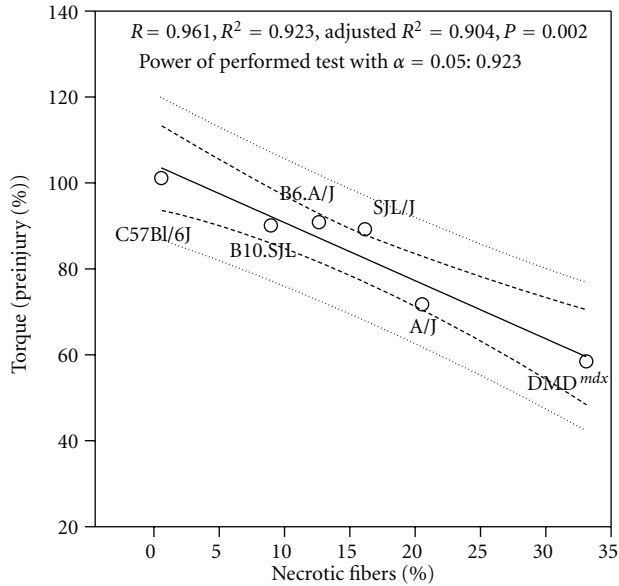


FIGURE 7: D3 contractile torque regressed on necrosis. A regression analysis of necrosis as the independent variable and D3 torque as the dependent variable showed a good linear fit, suggesting that the extent of necrosis is a reliable negative predictor of functional recovery at D3. The dashed and dotted lines represent the 95% confidence and prediction intervals, respectively.

than in muscles lacking dysferlin. Injury of  $DMD^{mdx}$  muscle is rapidly followed by significant myofiber degeneration (Figures 2 and 3).  $DMD^{mdx}$  mice are known to be much more susceptible than controls to injury induced by lengthening contractions [27–29], and the more pronounced effect of LSI on their muscles suggests that our injury model is indeed physiologically relevant to the study of sarcolemmal damage and repair.

Remarkably, however, although the physiological changes induced by LSI differ quantitatively from one strain to the other, they are qualitatively similar in all the strains we have studied, including control and dysferlinopathic strains. These are indistinguishable in their susceptibility to initial injury but differ 3 days later in necrosis and inflammation. By contrast,  $DMD^{mdx}$  mice are more susceptible to initial injury and also recover slowly. Consistent with this, regression analyses of loss of function on D3 following LSI, measured as torque loss, against the number of necrotic fibers on D3, show a strong linear correlation. Similarly, the number of macrophages in muscles on D3 post-LSI is a linear function of necrosis. In other studies, we have shown that the appearance of necrotic fibers precedes the appearance of macrophages in injured muscle by several hours (Roche JA and Bloch RJ, in preparation). These results therefore suggest that, despite the different mutations and genes affected in the dysferlinopathic and dystrophinopathic mice we have studied, and the different extents to which they recover contractile torque, the level of functional recovery and the extent of inflammatory cell infiltration are determined by the extent of myofiber necrosis. As macrophage infiltration in 3 dysferlinopathic strains (B6.A/J, SJL/J, and B10.SJL) is

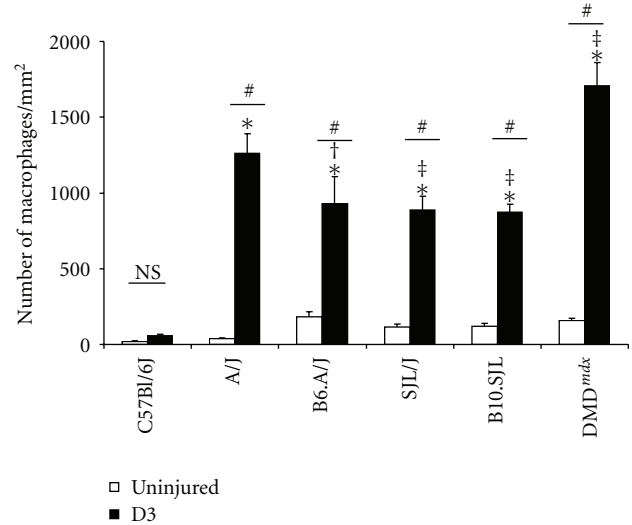


FIGURE 8: Quantitation of macrophages (CD68+ cells). We counted macrophages in cross sections of uninjured and injured TA labeled with antibodies to CD68 (see Figure 4). All dysferlin mutant strains showed elevated macrophage counts at D3 post-LSI, with A/J having significantly higher levels than the other strains.  $DMD^{mdx}$  muscle showed significantly higher macrophage infiltration than A/J. \*Significant difference compared to control strain at same time point. #Significant difference within same strain between uninjured and D3. †Significant difference compared to same *Dysf* mutation at same time point. ‡Significant difference compared to A/J at same time point.

significantly elevated at D3 despite near complete functional recovery and moderate levels of myofiber necrosis, our results support a role for increased infiltration of monocytes and macrophages in dysferlinopathies, as reported by others [32–34].

Most of our attention in these studies was focused on the properties of 4 different dysferlinopathic strains of mice, two carrying a retrotransposon in the dysferlin gene (A/J and B6.A/J) and two carrying a mutation in the *Dysf* gene that results in the deletion of an exon encoding 57 amino acids in one of dysferlin's C2 domains. We find that the key characteristics of the TA muscles of 12–16 wk old SJL/J and B10.SJL mice are low but readily detectable levels of central nucleation, necrosis, and macrophage infiltration in uninjured muscles, consistent with the early stages of myopathy reported in these mice at this age [9, 12]. Consistent with their shared phenotypes, both strains lose similar fractions of their initial torque after LSI, and they both recover to near complete levels, similar to their respective control strains, within 3 days. However, SJL/J muscles show greater numbers of CNFs before injury and greater necrosis 3 days after LSI, suggesting that genes other than dysferlin that are differentially expressed play a role in the myopathy that has been reported in SJL/J mice.

Our previous studies on the effect of LSI on A/J and A/WySnJ mice suggest that the presence or absence of dysferlin does not have an immediate effect on the ability of the sarcolemma to reseal, trapping 10 kDa fluorescein dextran in



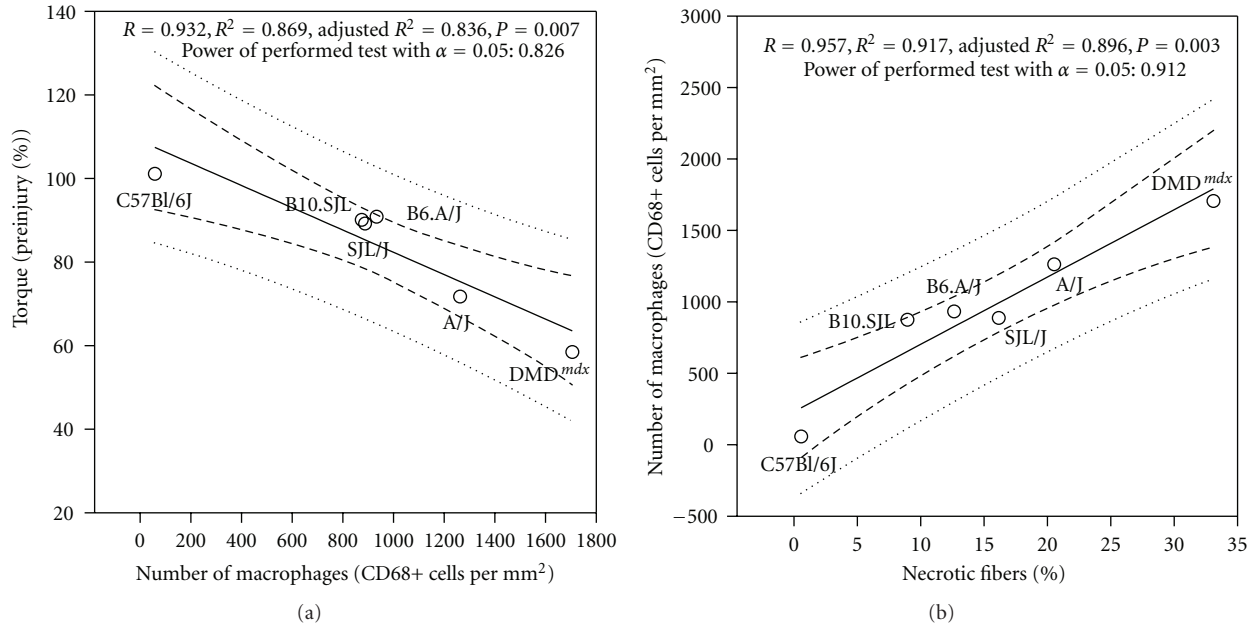


FIGURE 9: Regression analyses of contractile torque *versus* macrophage counts and macrophage counts *versus* necrosis. A regression analysis of the number of macrophages on D3 as the independent variable and torque at D3 as the dependent variable showed a good linear fit (a). Similarly, analysis of the number of necrotic fibers as the independent variable and the number of macrophages as the dependent variable also showed a good linear fit (b). The analyses suggest that that macrophage counts are a reliable negative predictor of functional recovery at D3 and that the number of necrotic fibers predicts the extent of macrophage invasion.

the myoplasm for several days after injury [18]. Rather, the damage to dysferlin-null muscle is a later event, subsequent to sarcolemmal resealing that manifests itself initially as a significant increase in necrosis followed later by increased inflammation. These changes are fully manifested by D3 after LSI, after which damaged myofibers are lost and replaced by myogenesis. Our results with the SJL/J and B10.SJL indicate that mutations in the dysferlin gene that reduce the levels of dysferlin protein in muscle to  $\sim 15\%$  of control levels or less [7, 12] are not sufficient to lead to the persistent torque deficit seen in A/J muscle 3 days after injury. However, despite the near complete functional recovery of SJL/J and B10.SJL muscles 3 days after injury, they still showed significantly elevated counts of necrotic fibers and macrophages, albeit less than A/J. They are nevertheless consistent with the idea that wild-type dysferlin is not needed for sarcolemmal membranes to reseal immediately after injury and protect fibers against rapid degeneration, as we see in dystrophin-deficient DMD<sup>mdx</sup> muscle. Indeed, the fact that these two strains of mice recover torque equally well after injury, and nearly as well as controls, suggests that the modified dysferlin they synthesize, albeit in very low amounts, may promote their accelerated recovery. Alternatively, the low levels of ongoing degeneration and regeneration, evident in uninjured muscles of these mice from their elevated numbers of CNFs, necrotic fibers, and macrophages, may accelerate their recovery from LSI, perhaps because they are already primed to regenerate.

Our current results with A/J and control, A/WySnJ, mice agree with our previous results, which indicate that A/J but not A/WySnJ muscle show more CNFs before injury, with a large increase in necrosis and inflammation 3 days after

injury [18]. CNFs only increase at later times, as A/J muscles regenerate [17, 18]. Consistent with this, torque is significantly depressed at D3 and recovers over 14 days after injury as myogenesis is activated to replace fibers lost as a result of the pathological changes that follow LSI.

Although they carry the same retrotransposon as A/J mice in their dysferlin gene and thus fail to express any dysferlin [7], B6.A/J mice respond differently to LSI. In particular, although they have similar levels of CNFs, necrotic fibers, and inflammation both before and after injury, the contractile torques we measure are highly variable. Moreover, their susceptibility to LSI appears lower than that of A/J mice (as well as SJL/J and B10.SJL), although the magnitude of this difference decreases as the sample size increases. We currently have no explanation for this variability. Until we can control it, however, we favor the A/J line for studying the role of dysferlin in muscles subjected to physiological injuries in living mice.

Our results suggest that A/J mice, and SJL/J and B10.SJL, are useful for studying different aspects of the muscular dystrophies linked to mutations in dysferlin. Because they show an earlier onset of myopathy, typical of the human disease, which is usually first observed in young adults, SJL/J and B10.SJL are potentially more useful for studying the progression of the disease *in vivo* and for testing the ability of drugs or other therapeutics to slow or block further myopathic changes. Given that C57Bl/10J mice are a better control for B10.SJL than SWR/J mice are for SJL/J, B10.SJL mice are a better choice for these types of experiments. Such studies will, however, require careful evaluation of the state of the muscles before any treatment, as they may already

be dystrophic. Because they show little pathology as young adults, but respond so differently to LSI than controls, A/J mice in our opinion remain very useful for studies designed to examine the specific role of dysferlin in muscles without the confounding effect of preexisting dystrophic changes. A/J mice also give insight into how apparently healthy muscles lacking dysferlin become dystrophic, thus modeling the subclinical phase of dysferlinopathies in humans. Given the differences between them, however, it seems advisable for laboratories to investigate both A/J and B10.SJL mice as they search for a cure for dysferlinopathies.

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