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1 **Degenerative and regenerative features of myofibers differ among skeletal muscles in a murine**
2 **model of muscular dystrophy**

3

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19

20 **Abstract**

21 Skeletal muscle myofibers constantly undergo degeneration and regeneration. Histopathological features
22 of 6 skeletal muscles (cranial tibial [CT], gastrocnemius, quadriceps femoris, triceps brachii [TB], lumbar
23 longissimus muscles, and costal part of the diaphragm [CPD]) were compared using
24 C57BL/10ScSn-*Dmd*^{mdx} (mdx) mice, a model for muscular dystrophy versus control, C57BL/10 mice.
25 Body weight and skeletal muscle mass were lower in mdx mice than the control at 4 weeks of age; these
26 results were similar at 6–30 weeks. Additionally, muscular lesions were observed in all examined skeletal
27 muscles in mdx mice after 4 weeks, but none were noted in the controls. Immunohistochemical staining
28 revealed numerous paired box 7-positive satellite cells surrounding the embryonic myosin heavy
29 chain-positive regenerating myofibers, while the number of the former and staining intensity of the latter
30 decreased as myofiber regeneration progressed. Persistent muscular lesions were observed in skeletal
31 muscles of mdx mice between 4–14 weeks of age, and normal myofibers decreased with age. Number of
32 muscular lesions was lowest in CPD at all ages examined, while the ratio of normal myofibers was lowest
33 in TB at 6 weeks. In CT, TB, and CPD, Iba1-positive macrophages, the main inflammatory cells in
34 skeletal muscle lesions, showed a significant positive correlation with the appearance of regenerating
35 myofibers. Additionally, B220-positive B-cells showed positive and negative correlation with
36 regenerating and regenerated myofibers, respectively. Our data suggest that degenerative and regenerative
37 features of myofibers differ among skeletal muscles and that inflammatory cells are strongly associated
38 with regenerative features of myofibers in mdx mice.

39

40 **Key words:** muscular dystrophy, mdx mouse, skeletal muscle remodeling, inflammation, triceps brachii,
41 costal part of the diaphragm

42

43 **Abbreviations:** CT, cranial tibial muscle; CPD, costal part of the diaphragm; GA, gastrocnemius muscle;
44 HE, hematoxylin-eosin; LL, lumbar longissimus muscle; MRF, myogenic regulatory factor; MT,
45 Masson's trichrome stain; QR, quadriceps femoris muscle; TB, triceps brachii muscle

46 **INTRODUCTION**

47 Skeletal muscles have high regenerative ability and satellite cells, also known as myogenic precursor
48 cells, play a key role in muscle regeneration (Chargé and Rudnicki 2004). Skeletal muscle remodeling,
49 which involves degeneration and regeneration of myofibers, is a constant process in all animals. Aging,
50 metabolic diseases, hereditary muscle diseases and neuromuscular diseases can affect the processes
51 involved in skeletal muscle remodeling, which lead to breakdown and atrophy of skeletal muscle. Muscle
52 repair is a multistep process that includes myofiber degeneration and regeneration (Le Grand and
53 Rudnicki 2007; Ten Broek et al. 2010; Tsivitse 2010). Following damage to myofibers, satellite cells are
54 activated and proliferate to give rise to a population of transient, amplifying myogenic cells called
55 myoblasts, which express myogenic regulatory factors (MRFs) such as myogenic differentiation 1 and
56 myogenic factor 5. Myoblasts subsequently express another MRF called myogenin, commit to terminal
57 differentiation, and fuse to reconstruct their host myofibers or to generate new myofibers and repair
58 damaged tissue (Tedesco et al. 2010).

59 Duchenne muscular dystrophy (DMD), the most common and severe type of muscular dystrophy in
60 humans, is an X-linked, recessive, lethal muscle wasting disease affecting approximately 1 in 3,500 boys
61 (Emery 1989). It is caused by mutations in the *DMD* gene, located on the X chromosome, which codes
62 for dystrophin, a membrane-associated structural protein. Homologues of *DMD* have been identified in
63 several animals including dogs (Cooper et al. 1988), cats (Carpenter et al. 1989) and mice (Bulfield et al.
64 1984; Pastoret and Seville 1995). *C57BL/10ScSn-Dmd^{mdx}* (*mdx*) mice do not produce dystrophin, due to
65 a point-mutation in *Dmd*. Though these mice do not represent a complete phenotypic model of DMD in
66 humans, they have been used as a representative animal model of DMD (Bulfield et al. 1984). These mice
67 show pathological changes in skeletal muscles, characterized by necrosis of skeletal muscle fibers
68 accompanied by regeneration (Bulfield et al. 1984; Pastoret and Seville 1995; Boland et al. 1995).

69 It has been observed that the pathological features of DMD differ among different types of skeletal
70 muscles. Skeletal muscles of the thoracic esophagus show no necrosis and no regenerating fibers in *mdx*

71 mice (Boland et al. 1995). However, large numbers of necrotic myofibers have been seen between 3 and 4
72 weeks of age in limb muscle (Shavlakadze et al. 2004), followed by regeneration of myofibers by 6 to
73 12 weeks of age (McGeachie et al. 1993). In contrast to the limb, muscles of the diaphragm show
74 progressive degeneration of myofibers (Stedman et al. 1991). Therefore, a comparison of
75 histopathological changes among the muscles of mdx mice would be useful to better understand the
76 pathological characteristics of DMD (Louboutin et al. 1993; Pastoret and Sebillé 1995; Boland et al.
77 1995).

78 Inflammation is a crucial process that is thought to both, exacerbate damage and necrosis as well as
79 promote regeneration in skeletal muscles. Inflammatory cells, especially neutrophils and macrophages,
80 mediate necrosis of muscle cells via oxidative damage both, *in vitro* (Pizza et al. 2001; McLoughlin et al.
81 2003; Nguyen and Tidball 2003) and *in vivo* (Pizza et al. 2005; Cheung and Tidball 2003; Nguyen and
82 Tidball 2003), with macrophages also performing their principal role of phagocytizing necrotic cells
83 (Almekinders and Gilbert 1986; Robertson et al. 1993; Lescaudron et al. 1999; Merly et al. 1999;
84 Mojumdar et al, 2014). Further, it has been proposed that an excessive inflammatory response can directly
85 damage myofibers under myopathic conditions such as dystrophies or myositis (Porter et al. 2002).
86 Importantly, DNA microarray studies of mdx muscle show that 30% of all differentially expressed genes
87 are associated with inflammation (Porter et al. 2002; Porter et al. 2004; Tidball 2005).

88 In this study, we demonstrated that degenerative and regenerative features of myofibers differ among
89 skeletal muscles in mdx mice, and that these differences are associated with immune cell infiltration into
90 the skeletal muscle lesions. Each skeletal muscle in the adult mammalian body has a different character
91 based on anatomical, physiological, and/or mechanical factors such as exercise load and posture of the
92 animal. The findings of this study shed light on the process of muscle remodeling and the pathological
93 differences among different types of skeletal muscles in DMD.

94 **MATERIALS AND METHODS**

95 *Experimental animals*

96 Animal experimentation was approved by the Institutional Animal Care and Use Committee of the
97 Graduate School of Veterinary Medicine, Hokkaido University (approval No. 13-0032). Male, mdx mice
98 were purchased from Central Institute for Experimental Animals (Kanagawa, Japan) and control, male
99 C57BL/10SnSlc (B10) mice were purchased from Japan SLC (Shizuoka, Japan). Mice were housed in the
100 animal facility of Graduate School of Veterinary Medicine, Hokkaido University. The body weight of B10
101 and mdx mice was measured at 1 (B10, mdx; n = 4, 19, respectively), 2 (n = 4, 17), 3 (n = 4, 17), 4 (n =
102 10, 17), 6 (n = 5, 12), 8 (n = 5, 11), 14 (n = 5, 10), and 30 (n = 4, 4) weeks of age. Skeletal muscles were
103 harvested at 4 (n = 4), 6 (n = 4), and 14 (n = 4) weeks of age. The genotype of mdx mice was analyzed by
104 TaqMan PCR (Applied Biosystems, Foster, CA, USA) targeting a point mutation in the *DMD* gene.
105 Experimental animals were handled in accordance with the Guide for the Care and Use of Laboratory
106 Animals, Graduate School of Veterinary Medicine, Hokkaido University (approved by the Association for
107 Assessment and Accreditation of Laboratory Animal Care International).

108

109 *Tissue preparation and microscopic observation*

110 The caudal vena cava was cut under deep anesthesia by an intraperitoneal injection (0.012–0.015
111 ml/g of body weight) of 2.5% Avertin, while 4% paraformaldehyde was perfused from the left ventricle.
112 Six skeletal muscles (cranial tibial muscle, CT; gastrocnemius muscle, GA; quadriceps femoris muscle,
113 QR; triceps brachii muscle, TB; lumbar longissimus muscle, LL; and costal part of the diaphragm, CPD)
114 were collected. The muscles from the left side of the body were fixed in 10% neutral buffer formalin for
115 32–48 hours for morphological examination, while those from the right side of the body were fixed in 4%
116 paraformaldehyde for 18–24 hours for immunohistochemical staining. After fixation, specimens were
117 dehydrated in graded alcohol and embedded in paraffin. Subsequently, 2- μ m-thick paraffin sections of
118 each skeletal muscle were deparaffinized, rehydrated, stained with hematoxylin-eosin (HE) and Masson's

119 trichrome stain (MT), and observed under a light microscope.

120

121 *Immunohistochemistry*

122 Immunohistochemical staining for embryonic myosin heavy chain (eMyHC), paired box 7 (PAX7),
123 Gr1 (Ly-6G), Iba1, B220, and CD3 was performed using 3- μ m-thick paraffin sections to detect
124 regenerating myofibers, satellite cells, granulocytes, pan-macrophages (including M1/M2 macrophages;
125 Pierezan et al, 2014), B-cells, and pan T-cells, respectively. The paraffin sections were deparaffinized and
126 antigen was retrieved. After cooling, slides were soaked in methanol containing 3% H₂O₂ for 20 minutes
127 at room temperature to remove internal peroxidase. After washing, sections were blocked with mouse
128 blocking reagent (Nichirei, Tokyo, Japan) for eMyHC and PAX7, and with 10% normal goat serum
129 (Nichirei, Tokyo, Japan) for Gr1, Iba1, B220, and CD3, for 60 minutes at room temperature and incubated
130 with primary antibodies overnight, at 4°C. After washing thrice with phosphate-buffered saline, sections
131 were incubated with secondary antibodies for 30 minutes at room temperature, washed, and incubated
132 with streptavidin-biotin complex (SABPO® kit; Nichirei, Tokyo, Japan) for 30 minutes. The sections
133 were then incubated with 3,3'-diaminobenzidine tetrahydrochloride-H₂O₂ solution. Finally, the sections
134 were lightly counterstained with hematoxylin. Details of the antigen retrieval method as well as the
135 source and dilution of the antibodies are listed in Table 1.

136

137 *Histoplanimetry*

138 Quantitative analysis of degenerative and regenerative myofibers was performed using ImageJ
139 (<http://rsb.info.nih.gov/ij/>) software, as previously described (Pastoret and Sebillé 1995). Briefly, opaque
140 as well as faintly colored fibers, frequently filled with phagocytes were classified as degenerating
141 myofibers. Small fibers in which, the cytoplasm takes on a uniformly bluish color, with central vesicular
142 nuclei were classified as regenerating myofibers. Fibers with non-peripheral nuclei and eosinophilic
143 cytoplasm were classified as regenerated myofibers. All others were classified as normal myofibers. All

144 myofibers in transverse sections of each skeletal muscle were counted (at least 2,000 myofibers per
145 specimen) and classified into 1 of the 4 types of myofibers described above. Based on
146 immunohistochemical staining, the number of Gr1-, Iba1-, B220-, and CD3-positive cells in each skeletal
147 muscle was counted, and the total area of transverse sections of each skeletal muscle was then measured.
148 Finally, the number of immune-positive cells was divided by the total area of transverse sections of each
149 skeletal muscle, and these values were expressed as cell number per unit area (number/mm²).

150

151 *Statistical analysis*

152 All numerical results are presented as mean \pm standard error (SE) and analyzed using non-parametric
153 methods. The Mann-Whitney *U*-test was used to compare 2 groups ($P < 0.05$). The Kruskal-Wallis test
154 was used to compare 3 or more groups, and multiple comparisons were performed using Scheffé's
155 method when a significant difference was observed ($P < 0.05$). Correlation between myofibers exhibiting
156 histopathological changes and immune cell numbers in the skeletal muscle lesions was analyzed by
157 Spearman's correlation coefficient test (ρ) ($P < 0.05$).

158 **Results**

159 *Body weight and skeletal muscle mass in mdx and B10 mice*

160 Changes in body weight and skeletal muscle mass were compared between mdx and B10 mice from
161 1 to 30 weeks of age. Body weight was similar between the 2 groups up to 3 weeks of age. However, at
162 4 weeks, the body weight of mdx mice was significantly lower than that of B10 mice (Figure 1A). From 6
163 weeks onwards, body weight was similar again between the two groups. Gross anatomical and
164 histological examination at 4 weeks of age revealed that the muscle mass of mdx mice was markedly
165 lower compared to that of B10 mice. Accordingly, the skeletal muscle area in the transverse sections of all
166 examined skeletal muscles was reduced in mdx mice than in B10 mice at 4 weeks of age (Figure 1B).
167 Muscular histopathological changes, such as degenerative and atrophied myofibers, were widely but
168 focally observed in mdx mice, but not in B10 mice (Figure 1B). However, at 6 weeks of age, the skeletal
169 muscle areas of mdx mice seemed to increase to almost equal levels to those in B10 mice (Figure 1C). In
170 mdx mice, myofibers with non-peripheral nuclei occupied the observed area at 6 weeks (Figure 1C). In
171 addition to the results of body weight change and muscle features (Figure 1), we considered previous
172 reports (Shavlakadze et al. 2004; McGeachie et al. 1993) and examined ages and divided the mdx mouse
173 group into the muscle degenerative phase (4 weeks of ages), muscle regenerative phase (6 weeks onward),
174 and the sexually matured and muscle regenerated phase (14 weeks).

175

176 *Histopathological features of skeletal muscle in mdx and B10 mice*

177 We analyzed the histopathological changes shown in Figure 1B in more detail. As shown in Figure 2,
178 B10 mice showed no histological changes in any of the examined muscles. In mdx mice, on the other
179 hand showed no histopathological changes in skeletal muscles at 2 weeks of age, and very few changes at
180 3 weeks (data not shown). However, severe histopathological changes were observed from 4 weeks of age
181 (Figure 2). Muscular lesions were observed focally in all mdx mice at all examined ages. These lesions
182 consisted of 3 types of myofibers: degenerative myofibers (arrows in Figure 2), regenerating myofibers

183 (small arrowheads in Figure 2), and regenerated myofibers (large arrowheads in Figure 2). Although these
184 muscular lesions were observed in all examined muscles of mdx mice, the number of degenerative
185 myofibers tended to be lower in CPD compared to other skeletal muscles (Figure 2). These results suggest
186 that the progression of histopathological changes in mdx mice may differ among skeletal muscles.

187 To further investigate regenerative features of myofibers in mdx mice, immunohistochemical
188 staining was carried out. Numerous PAX7-positive muscle satellite cells surrounded eMyHC-positive
189 regenerating myofibers in skeletal muscles of mdx mice (Figure 3A–F), and the number of the former and
190 the staining intensity of the latter gradually decreased with the progression of myofiber regeneration
191 (Figure 3A–C and G–L).

192

193 *Histopathological changes in different skeletal muscles of mdx mice*

194 To compare the muscular lesions among examined skeletal muscles in mdx mice, we compared the
195 ratio of the number of fibers classified as normal, degenerative, and centronucleated
196 (regenerating/regenerated) to the total number of muscle fibers in each skeletal muscle examined
197 (Figure 4). Although all examined skeletal muscles contained muscular lesions during the observation
198 period, the ratio of each type of myofiber differed among skeletal muscles. The ratios of normal and
199 degenerative myofibers were larger and smaller, respectively, in CPD compared with other muscles at 4
200 weeks of age (Figure 4A and B). The ratios of normal myofibers also tended to be larger in CPD than in
201 the other muscles at 6 and 14 weeks of age (Figure 4A). Further, the ratio of centronucleated myofibers,
202 indicating regenerating or regenerated myofibers, tended to be smaller in CPD than in the other muscles
203 (Figure 4C). Significant differences between CPD and the other muscles were observed in terms of ratio
204 of regenerated myofibers and that of regenerating myofibers (Figure 4D and E). At 6 weeks of age, the
205 ratio of normal myofibers was significantly smaller in TB as compared to that in CT, GA, and CPD. In
206 addition to these variations among different skeletal muscles, significant differences were noted in
207 muscular lesions with respect to ratios, within the same skeletal muscle. The ratio of normal and

208 centronucleated myofibers, especially regenerated myofibers, were smaller and larger, respectively, at 14
209 as compared to 4 weeks of age, in all examined muscles (Figure 4A, C, and E). These results indicate that
210 the pathological features of muscular lesions differed among skeletal muscles and with age. In particular,
211 CPD and TB tended to show milder and severer lesions, respectively, compared with other examined
212 skeletal muscles, in mdx mice.

213

214 *Distribution of immune cells in skeletal muscle lesions*

215 Since morphological analysis using HE and/or MT staining indicated the presence of macrophages
216 and granulocytes (especially neutrophils) in the muscular lesions (Figure 2 and 3D), the distribution of
217 immune cells was analyzed by immunohistochemistry. Histopathological analyses described above
218 indicated that TB and CPD showed different features of muscular lesions compared with the other
219 skeletal muscles examined. Therefore, immunohistochemical analysis was performed for TB, CPD, and
220 CT muscles, where CT served as control. Immunohistochemical staining revealed the presence of Iba1-,
221 Gr1-, B220-, and CD3-positive cells in the skeletal muscle lesions of mdx mice (Figure 5A–D). All
222 positive reactions were counted and normalized by the area of each skeletal muscle. Iba1- and
223 Gr1-positive cells were high in TB at 4 weeks of age and low in TB and CPD at 14 weeks of age, but no
224 significant differences were noted in any skeletal muscle with respect to age (Figure 5E and F).
225 B220-positive cells in CT and TB, whereas CD3-positive cells in TB were significantly higher at 4 weeks
226 of age as compared to those at 6 and 14 weeks of age (Figure 5G and H). A significant difference in the
227 number of immune cells in the examined skeletal muscles at the same age was observed only in CD3 at
228 14 weeks of age. CPD showed a large number of CD3-positive cells compared to CT, at 14 weeks of age
229 (Figure 5H). The number of Iba1-positive cells was the highest of the 4 immune cells examined, in all
230 skeletal muscles at the same age (Figure 5E).

231

232 *Correlation between histopathological changes in skeletal muscles and immune cell numbers*

233 As shown in Figure 6, a significant correlation was observed between Iba1-positive cells and
234 regenerating myofibers (Figure 6B), between Gr1 and regenerated myofibers (Figure 6F), and between
235 B220-positive cells and regenerated myofibers (Figure 6I).

236

237 **Discussion**

238 The present study demonstrated that degenerative and regenerative features of myofibers differ
239 among skeletal muscles in mdx DMD model mice. Body weight of mdx mice was significantly lower
240 than that of B10 mice at 4 weeks of age, but was similar at 6 weeks. Myofiber necrosis has been
241 previously observed in mdx mice at 3 weeks of age, followed by myofiber regeneration (Coulton et al.
242 1988; McGeachie et al. 1993; Grounds and Torrisi 2004). Additionally, a large number of necrotic
243 myofibers have been observed between 3 and 4 weeks of age in CT muscle (Shavlakadze et al. 2004).
244 After onset of acute myofiber necrosis, degeneration of skeletal muscle decreases accompanied by
245 regeneration of myofibers and is at a low level by 6 to 12 weeks of age (McGeachie et al. 1993). In the
246 present study, the skeletal muscle area was smaller in mdx mice than in B10 mice in transverse sections
247 of all examined skeletal muscles at 4 weeks of age, but was comparable at 6 weeks. This recovery of
248 the skeletal muscle area is thought to be the result of acute regeneration of myofibers. Further, Pastoret
249 and Seville (1995) compared the body weight and CT muscles of mdx and B10 mice from 2 to 104
250 weeks of age, resulting that mdx mice showed higher body weight than B10 mice from 13 to 26 weeks
251 of age, due to hypertrophy of mdx skeletal muscle. Therefore, hypertrophy of skeletal muscle could be
252 one of the morphological features of myofiber regeneration in mdx mice. A significant difference in
253 body weight of mdx and B10 mice at 4 weeks of age was observed in the present study, which may be
254 due to degeneration of skeletal muscles in mdx mice.

255 From 4 weeks of age onwards, skeletal muscle lesions containing degenerating, regenerating, or
256 regenerated myofibers were observed in mdx mice. Different muscles have been analyzed in mdx mice
257 after exercise (Weller et al. 1990; Brussee et al. 1997; Granchelli et al. 2000; De Luca et al. 2005),
258 administration of drugs (Hodgetts et al. 2006), engraftment of somatic cells (Hindi et al. 2013; Boldrin
259 et al. 2015), and gene modification (Li et al. 2009; Heydemann et al. 2012). The results obtained and
260 the skeletal muscles analyzed are different in these previous reports. The present study compared 6
261 skeletal muscles: CT, GA, QR, TB, LL, and CPD at 4, 6 and 14 weeks of age and found that the extent

262 of degeneration and regeneration differs among skeletal muscles with age, in mdx mice. CPD tended to
263 show less pathological changes than other skeletal muscles in mdx mice during the examined period. A
264 similar observation was reported by Louboutin et al., who demonstrated that histopathological changes
265 in the mdx diaphragm are rare before 25 days of age, whereas the degeneration of myofibers begins at
266 21-25 days of age in the hind limb muscles (extensor digitorum longus: EDL, CT, and soleus).
267 Additionally, regenerating fibers with central nuclei were fewer in the diaphragm than in hind limb
268 muscles during the observation period of 30 to 270 days of age (Louboutin et al. 1993). Boland et al.
269 also showed a lower number of myofibers with central nuclei and a higher number of myofibers with
270 peripheral nuclei in the diaphragm than in CT and rectus abdominis muscles of mdx mice during 12-18
271 months of age (Boland et al., 1995). Although the pathological implications of nuclear position in
272 muscle diseases might be debatable, these data and a recent study showed skeletal muscle fibers with
273 central nuclei as regenerated myofibers (Boldrin et al., 2015); thus we considered that central nuclei
274 could be used as a marker of regeneration. In the present study, CPD tended to show a higher ratio of
275 normal fiber as compared to the other skeletal muscles, especially at 4 weeks of age. Regeneration of
276 myofibers begins after degeneration and the number of regeneration fibers increased clearly with age in
277 CPD of mdx mice. This indicates that CPD shows late-onset degeneration of myofibers. However,
278 Louboutin *et al.* also showed that fibrosis, occasionally including adipose tissue replacement, is
279 observed at 270 days of age in the diaphragm, but was less-frequent in hind limb muscles (Louboutin et
280 al. 1993), indicating that CPD might have lower regenerative ability as compared to other skeletal
281 muscles. Fiber-type transition is a pathological response against contraction-induced injury in DMD
282 (Gehrig et al. 2010). However, Schneider et al. observed the diaphragm as a severely affected
283 muscle in DMD and examined the fiber-type switch compared with quadriceps (a fast-twitch
284 muscle) and soleus (a slow-twitch muscle) in 2-month-old mouse models of DMD. They reported
285 fiber-type transition from fast-fiber (type 2b) to slow-fiber (type 1 and type 2a) in all of the 3
286 muscles; however, type 1 MyHC and eMyHC are not co-expressed, suggesting that regeneration

287 and fiber-type switch are two independent mechanisms in the dystrophic muscles (Schneider et al.,
288 2013).

289 In addition, the present study shows that increased muscular lesions are observed in TB compared
290 to the other skeletal muscles at 6 weeks of age. Brussee *et al.* also reported that TB of mdx mice
291 showed more atrophied myofibers than CT, EDL, soleus, GA, biceps brachii, and diaphragm muscles
292 under sedentary conditions (Brussee et al. 1997). Interestingly, they also showed that the severity of
293 muscle damage among skeletal muscles differed after 3 days of downhill running exercise compared to
294 sedentary conditions (Brussee et al. 1997). The present study shows that the degree of degeneration and
295 regeneration, involving damage to myofibers or subsequent reactions of muscular satellite cells or
296 myoblasts, differs among skeletal muscles, which may have an effect on the phenotypes of mdx mice.

297 The difference in muscle pathological features in mdx mice has been examined in several previous
298 reports (Louboutin et al. 1993; Brussee et al. 1997); however, their inflammatory features in different
299 muscles are scarcely addressed. The present study demonstrated that Iba1-positive macrophages are the
300 major inflammatory cells in skeletal muscle lesions that positively correlate with the number of
301 regenerating myofibers, whereas Gr1-positive granulocytes, mainly neutrophils, negatively correlate
302 with regenerated myofibers in mdx mice. After muscle injury caused by trauma such as extensive
303 physical activity, or by innate genetic defects, inflammatory cells such as neutrophils and macrophages
304 infiltrate into the lesions (Belcastro et al. 1996). Necrosis of myofibers is the initial event in skeletal
305 muscle degeneration, accompanied by the activation of mononucleated cells, principally inflammatory
306 and myogenic cells. Neutrophils are the first inflammatory cells to infiltrate the injured muscle, with a
307 significant increase in their numbers as early as 1–6 h after myotoxin- or exercise-induced muscle
308 damage (Orimo et al. 1991; Fielding et al. 1993). Some reports revealed that prevention of neutrophil
309 infiltration after injury reduces force deficits and histological damage to muscle fibers (Walden et al.
310 1990; Brickson et al. 2003; Pizza et al. 2005; Lockhart and Brooks 2008), suggesting that neutrophils
311 contribute to muscle fiber damage. In the present study, although neutrophils did not show a correlation

312 with degenerating myofibers, a negative correlation with regenerated myofibers was noted, indicating
313 that neutrophils decrease with progression of regeneration. After neutrophil infiltration, macrophages
314 become the predominant inflammatory cell type within the site of injury (Orimo et al. 1991; Tidball
315 2005). Macrophages, especially the M2-type, infiltrate the injured site to phagocytose cellular debris
316 and may affect other aspects of muscle regeneration by activating myogenic cells (Almekinders and
317 Gilbert 1986; Robertson et al. 1993; Lescaudron et al. 1999; Merly et al. 1999). Muscle degeneration is
318 followed by the activation of a muscle repair process. Myogenic cells differentiate and fuse to existing
319 damaged fibers for repair or to one another for new myofiber formation (Snow 1977; Snow 1978; Darr
320 and Schultz 1987). Muscle regeneration is slower in older animals, which coincides with weakened
321 phagocytosis activity by inflammatory cells (Zacks and Sheff MF 1982; Grounds 1987). In addition,
322 mouse strains with slower rates of phagocytic removal of muscle debris show slower rates of muscle
323 regeneration (Grounds 1987). No significant difference in Iba1- and Gr1-positive cell infiltration was
324 observed among muscles in the present study; however, many studies support the hypothesis that
325 phagocytosis is a necessary feature of muscle repair, and that macrophages and neutrophils play an
326 important role in this process.

327 The present study also revealed that B220-positive, B cells were negatively correlated with
328 regenerated myofibers. Several studies suggest that B- and T-cells contribute to the development of
329 muscle fibrosis in aged (> 12 months) SCID-mdx (Farini et al. 2007) and nu/nu-mdx mice (Morrison et
330 al. 2000). The absence of B and T cells results in reduced fibrosis, accompanied by a reduction of
331 transforming growth factor- β 1 (Farini et al. 2007). In the present study, although the number of B
332 and/or T cells was significantly lower than that of macrophages (data not shown) and slightly lower
333 than that of neutrophils, in all examined skeletal muscles at each age, numbers of both B and T cells
334 were significantly higher in TB at 4 weeks of age compared to that at 6 and 14 weeks of age, suggesting
335 their importance in the early stages of muscular dystrophy in mdx mice.

336 In conclusion, the degenerative and regenerative features of myofibers in mdx mice differ among

337 skeletal muscles. Inflammatory cells, especially phagocytes and B cells, are strongly associated with
338 the regenerative features of myofibers. Though the triggers for these pathological differences are
339 unknown, anatomical, physiological, and/or mechanical factors such as exercise load may affect these
340 differences in remodeling processes among examined skeletal muscles. Further research involving the
341 study of muscle cells using cell-specific markers and quantification of MRFs in skeletal muscles, is
342 required to further understand muscular remodeling.

343

344 **Authors' contributions**

345 YK conceptualized the study. TI, OI, TN, YE, and YK designed the experiments. TI performed the
346 experiments and analyzed the data. TI, OI, and YK drafted the manuscript.

347

348 **Disclosures**

349 The authors declare no conflicts of interest.

350

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490

491 **Figure Legends**

492 **Fig. 1** Age-dependent changes in phenotypes of B10 and mdx mice.

493 **(A)** Comparison of body weights between B10 and mdx mice. Values = mean \pm SE. B10: $n \geq 4$; mdx: n
494 ≥ 10 . *: $P < 0.01$ vs B10 at same age, Mann-Whitney *U*-test.

495 **(B)** Transverse sections of triceps brachii muscle from B10 and mdx mice stained with Masson's
496 trichrome stain at 4 and 6 weeks of age. Bars = 1 mm (low magnification), 100 μ m (high magnification).

497

498 **Fig. 2** Comparison of myofibers between B10 (left) and mdx mice (right).

499 Transverse sections of triceps brachii muscle (TB) and costal part of the diaphragm (CPD) stained with
500 Masson's trichrome stain at 6 weeks of age. Squared areas within low magnification images are

501 magnified in the right panels. B10 mice show no histopathological change in either TB or CPD. In mdx
502 mice, degenerative myofibers (arrows, magnified panel of TB) are characterized by faint-colored and
503 disrupting myofibers. Regenerating myofibers (small arrowheads, magnified panel of TB) are small and
504 basophilic myofibers with central nuclei. Regenerated myofibers (large arrowheads, magnified panel of
505 TB and CPD) have eosinophilic large myofibers and non-peripheral (including central) nuclei in mdx
506 mice. Bars = 100 μ m (low magnification), 20 μ m (high magnification). w: weeks of age.

507

508 **Fig. 3** Regenerative features of skeletal muscle lesions in mdx mice.

509 Transverse sections of the same area of triceps brachii muscle in mdx mice at 4 weeks of age.
510 Hematoxylin and eosin staining (**A, D, G, and J**) and immunohistochemical staining for embryonic
511 myosin heavy chain (eMyHC) (B, E, H, and K) and paired box 7 (PAX7) (**C, F, I, and L**). Numerous
512 PAX7-positive muscle satellite cells surround eMyHC-positive regenerating myofibers in skeletal
513 muscles of mdx mice (**A–F**), and the number of the former and the staining intensity of the latter
514 gradually decrease with progression of myofiber regeneration (**A–C and G–L**). Bars = 100 μ m (low
515 magnification), 20 μ m (high magnification).

516

517 **Fig. 4** Ratio of degenerative and regenerative myofibers in skeletal muscles in mdx mice.

518 Myofibers classified into normal myofibers (**A**), degenerative myofibers (**B**), centronucleated myofibers
519 (**C**), regenerating myofibers (**D**), or regenerated myofibers (**E**). The sum of values of regenerating
520 myofibers and regenerated myofibers indicates the values of centronucleated myofibers. CT, cranial tibial
521 muscle; GA, gastrocnemius muscle; QR, quadriceps femoris muscle; TB, triceps brachii muscle; LL,
522 lumbar longissimus muscle; CPD, costal part of the diaphragm. Statistically significant differences among
523 muscles from mice of the same age are indicated by different letters (C, G, Q, T, and D indicating CT, GA,
524 QR, TB and CPD, respectively). Significant age differences between samples of the same muscle are
525 indicated by symbols (* and †). Analyses were performed using Kruskal-Wallis test, followed by Scheffé's

526 method for multiple comparisons when a significant difference is observed ($P < 0.05$). Values = mean \pm
527 SE; 4 w, n = 4; 6 w and 14 w, n = 3. w: weeks of age

528

529 **Fig. 5** Infiltration of immune cells in skeletal muscle lesions in mdx mice.

530 (A–D) Immunohistochemical staining for Iba1, Gr1, B220, and CD3 in triceps brachii muscle of mdx
531 mice at 6 weeks of age. Bars: 100 μ m (panels), 20 μ m (insets).

532 (E–H) Comparison of the number of inflammatory cells in each skeletal muscle for mice at different ages.

533 See Figure 4 legend for abbreviations. Statistically significant differences among muscles from mice of
534 the same age are indicated by different letters (C, G, Q, T, and D indicating CT, GA, QR, TB, and CPD,
535 respectively). Significant age differences between samples of the same muscle are indicated by symbols

536 (* and †). Analyses were performed using Kruskal-Wallis test, followed by Scheffé’s method for multiple

537 comparisons when a significant difference was observed ($P < 0.05$). Values = mean \pm SE. 4 w, n = 4; 6 w

538 and 14 w, n = 3. w: weeks of age

539

540 **Fig. 6** Correlation between inflammatory cell infiltration and ratio of degenerating, regenerating, and
541 regenerated myofibers in mdx mice.

542 Graph showing Spearman’s rank correlations between the density of inflammatory cells (Iba1: a-c, Gr1:

543 d-f, B220: g-i, and CD3: j-l) and the ratio of degenerating (a, d, g and j), regenerating (b, e, h and k), and

544 regenerated (c, f, i and l) myofibers. *: $P < 0.05$, Spearman’s rank correlation test; ρ : Spearman’s rank

545 correlation coefficient. n = 29.

546

547 **Table 1** Antibodies, working dilutions, and methods for antigen retrieval

Antibody	Source	Dilution	Antigen retrieval	Heating condition
Mouse anti-eMyHC	DSHB (Iowa, USA)	1:900	20 mM Tris-HCl (pH 9.0)	105°C, 20 minutes
Mouse anti-PAX7	DSHB (Iowa, USA)	1:200	20 mM Tris-HCl (pH 9.0)	105°C, 20 minutes
Rabbit anti-Iba1	Wako (Osaka, Japan)	1:2000	0.1% pepsin/ 0.2 N HCl	37°C, 5 minutes
Rat anti-Gr1	R and D system (Minneapolis, USA)	1:800	0.1% pepsin/ 0.2 N HCl	37°C, 5 minutes
Rat anti-B220	Cedarlane (Ontario, Canada)	1:1600	20 mM Tris-HCl (pH 9.0)	105°C, 20 minutes
Rabbit anti-CD3	Nichirei (Tokyo, Japan)	1:500	20 mM Tris-HCl (pH 9.0)	105°C, 20 minutes

Fig. 2 Comparison of myofibers between B10 (left) and mdx mice (right).

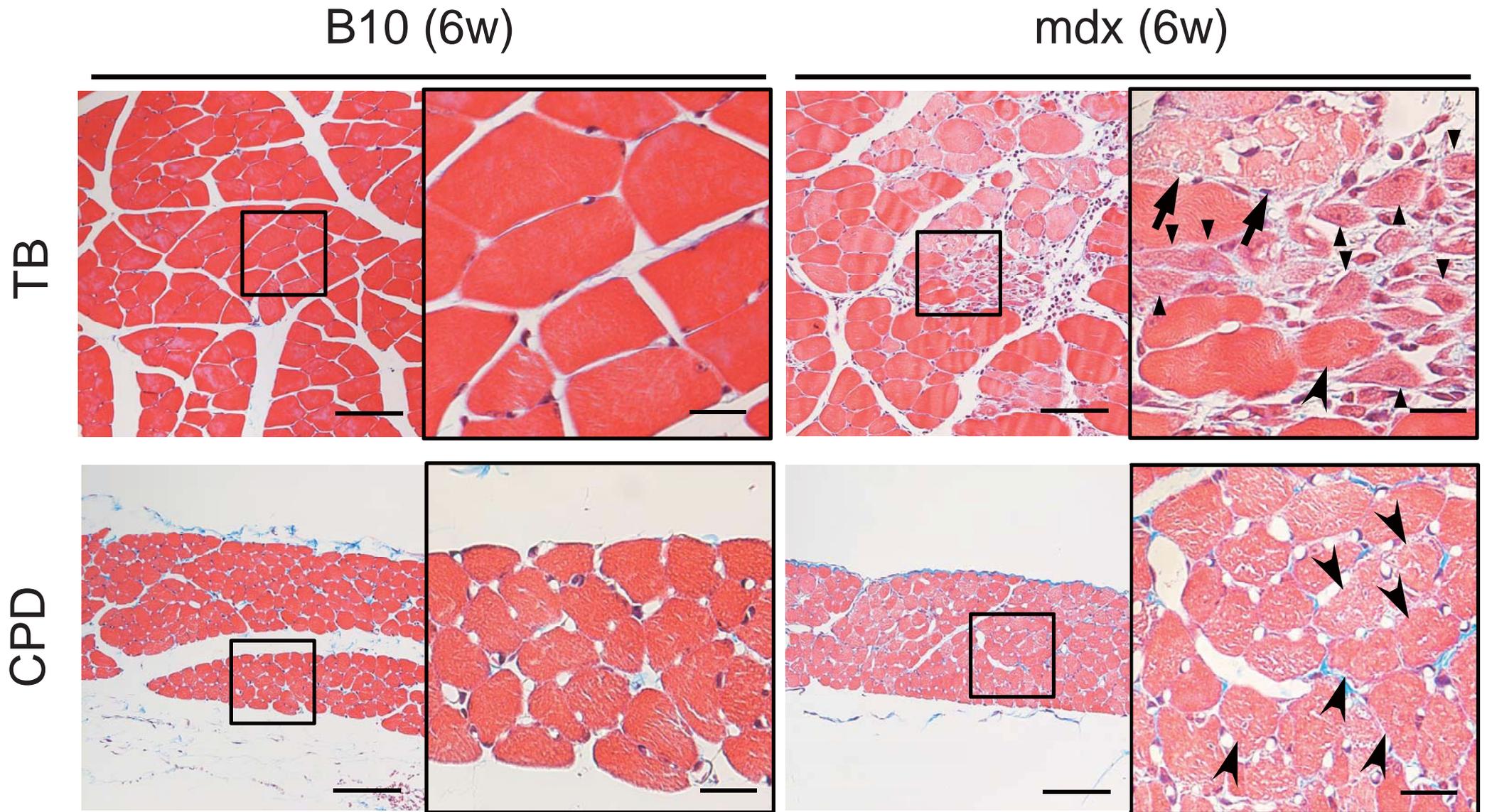
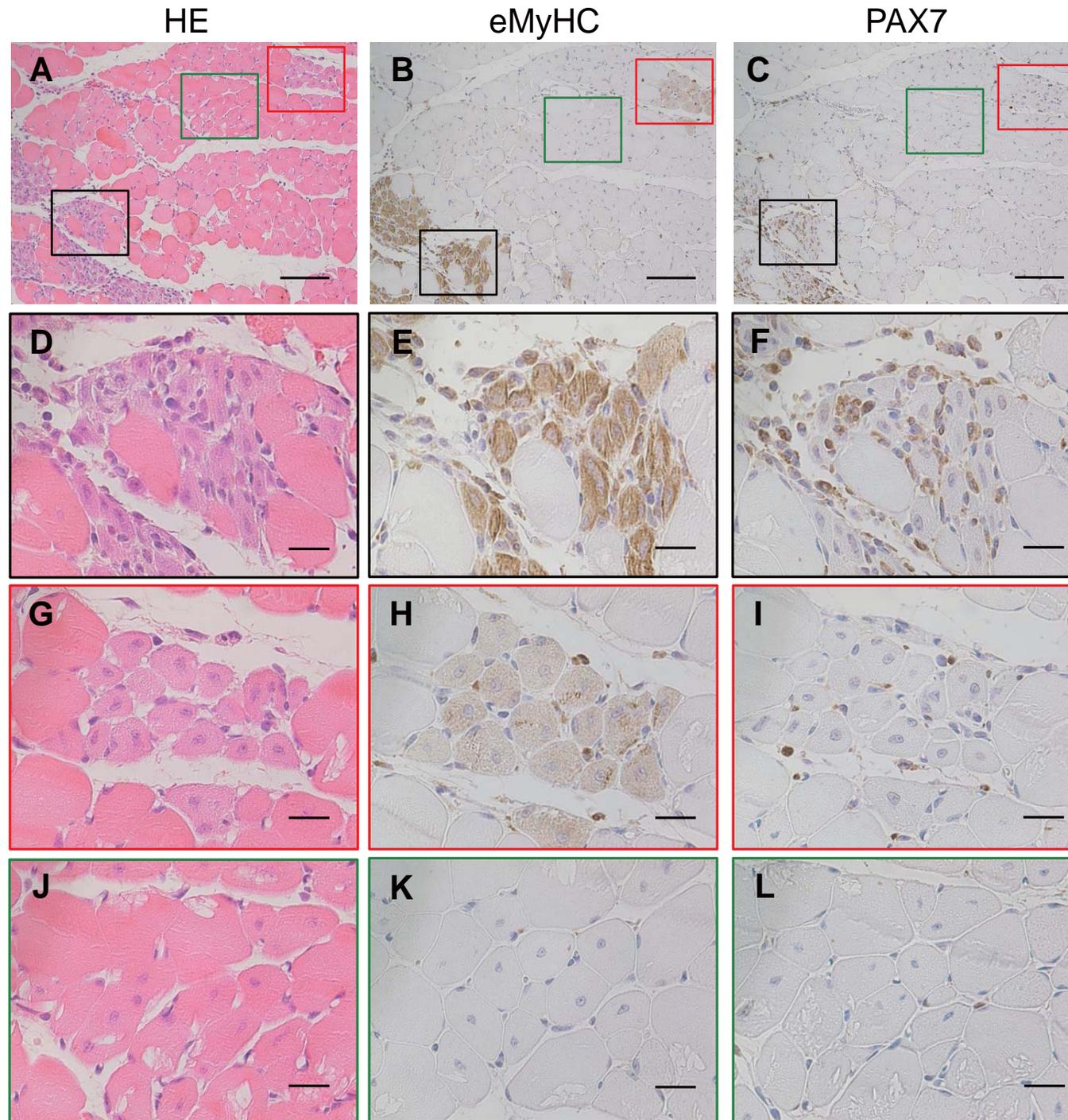
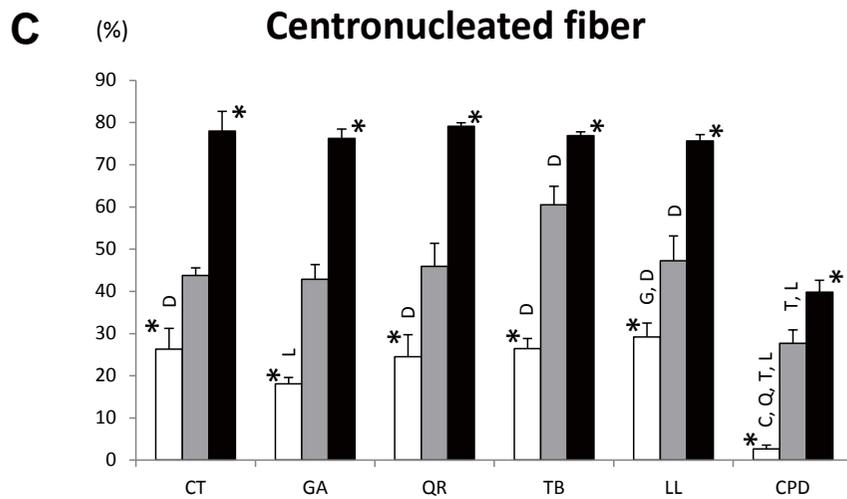
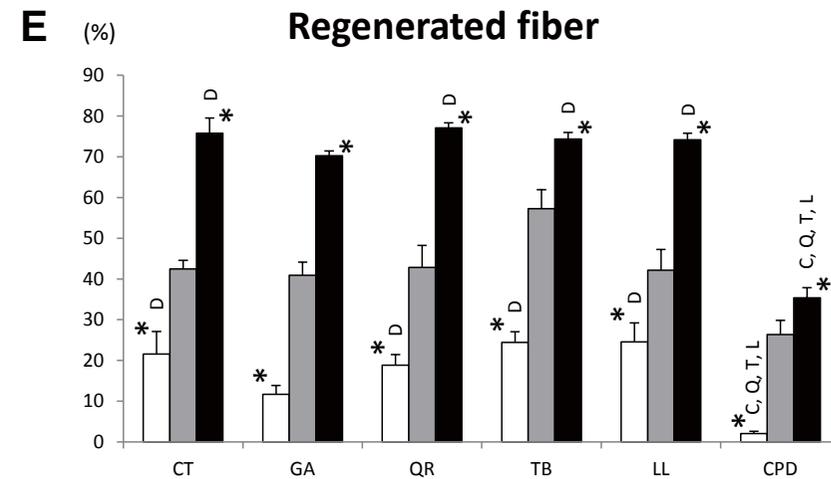
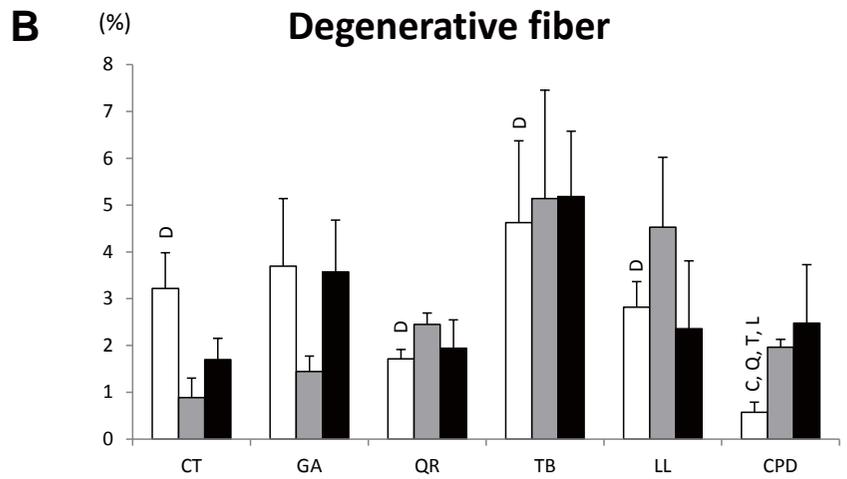
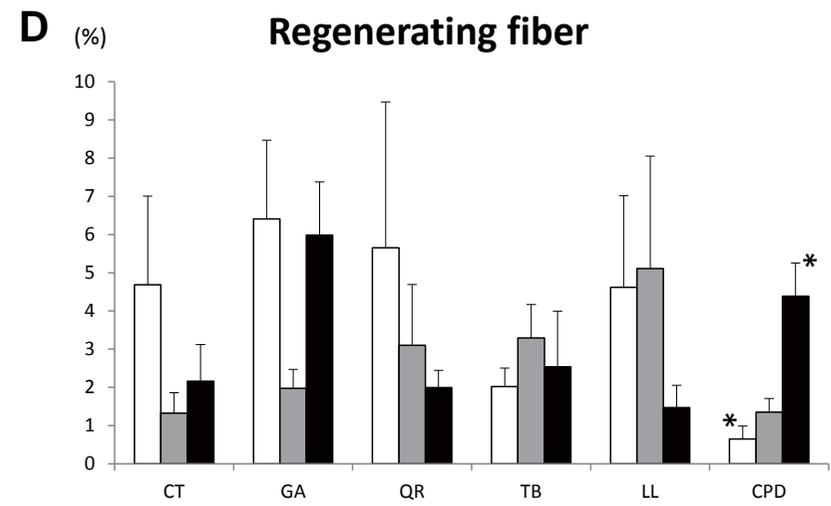
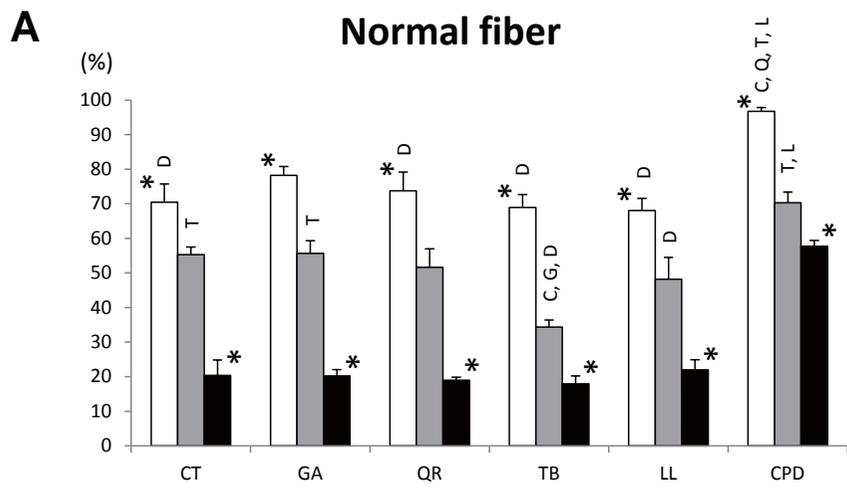


Fig. 3 Regenerative features of skeletal muscle lesions in mdx mice.





□ 4w ■ 6w ■ 14w

Fig. 4 Ratio of degenerative and regenerative myofibers in skeletal muscles in mdx mice.

Fig. 5 Infiltration of immune cells in skeletal muscle lesions in mdx mice.

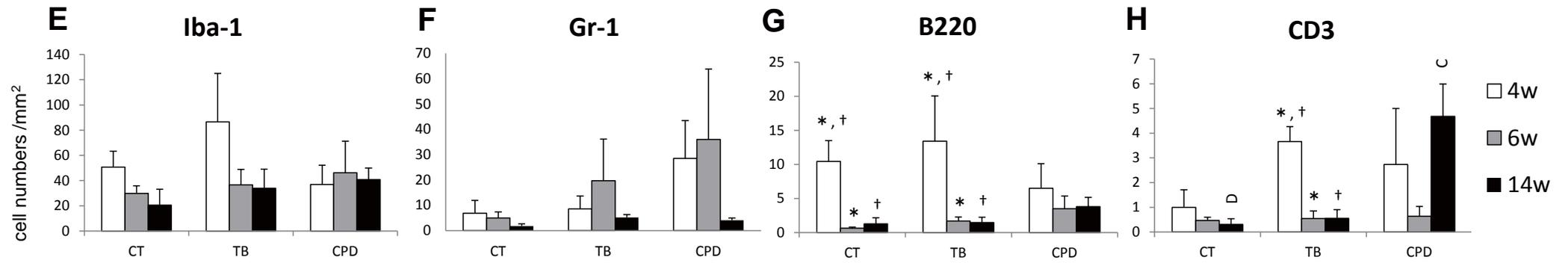
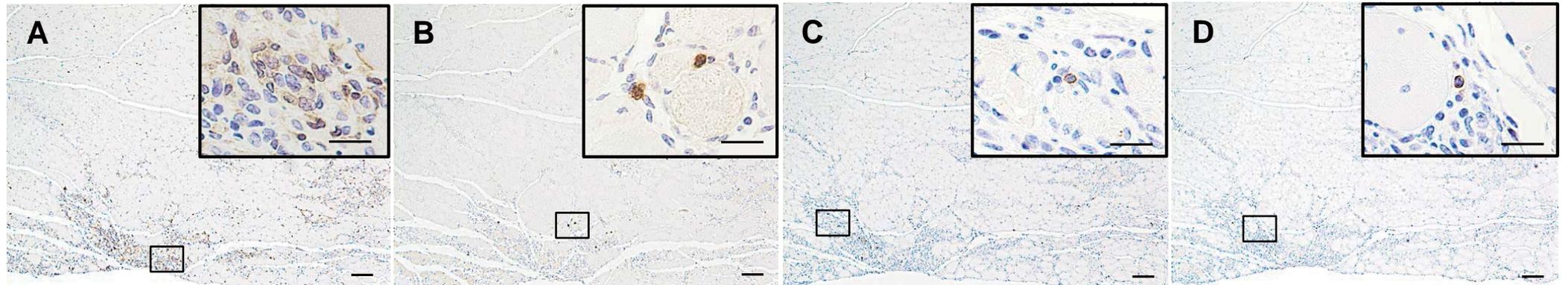


Fig. 6 Correlation between inflammatory cell infiltration and ratio of degenerating, regenerating, and regenerated myofibers in mdx mice.

