



Title	Effects of autoimmune abnormalities on skeletal muscle regeneration after needle puncture in mice
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1 **Effects of autoimmune abnormalities on skeletal muscle regeneration after needle**
2 **puncture in mice**

3

4 **Short title:** Punctured muscle injury and regeneration in mice with autoimmune abnormalities

5

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17

18

19 **Abstract**

20 Regeneration of injured skeletal muscles is supported by the activation of satellite cells, and
21 excessive traumatic injuries may trigger abnormal processes, such as fibrosis. Because the
22 participation of immune cells is crucial during skeletal muscle repair, systemic autoimmune
23 diseases impair their regeneration. This study focused on a traumatic injury by injection and
24 investigated the effect of autoimmune diseases on skeletal muscle regeneration. Male mice of
25 MRL/MpJ-*Fas*^{lpr/lpr} and MRL/MpJ (6–7 months old) were used for autoimmune disease and
26 healthy groups. The abdominal walls punctured by a needle were histologically analyzed at 1,
27 3, and 8 days post-injection. In both groups, injured skeletal muscle tissues showed necrosis
28 and inflammatory cell infiltrations on day 1, increased cell density at 3 days, and regenerative
29 myotubes with central nuclei without fibrosis at 8 days. Gr-1⁺ neutrophils at injured skeletal
30 muscle were abundant at 1 day, and then substantially decreased starting from 3 days in both
31 groups. The number of CD3⁺ T cells was remarkably higher in MRL/MpJ-*Fas*^{lpr/lpr} than that in
32 MRL/MpJ at 1 day, and a similar tendency was observed in B220⁺ B cells. The numbers of
33 IBA1⁺ macrophages and bromodeoxyuridine-incorporating cells tended to be higher at 3 days,
34 and those of the latter, mainly proliferating paired-box-7⁺ satellite cells, showed significance
35 at other time points and negatively correlated with the autoimmune disease indices, such as
36 spleen weights or serum autoantibody level. Thus, this result suggested that injured skeletal
37 muscle by minor trauma is normally regenerated regardless of the effects of autoimmune
38 diseases, although lymphocyte infiltrations during these processes were more severe in
39 MRL/MpJ-*Fas*^{lpr/lpr}.

40 **Keywords:** autoimmune disease, MRL/MpJ-*Fas*^{lpr/lpr}, skeletal muscle, injection, muscle
41 regeneration, histopathology

42

43 **Impact statement**

44 Mild traumatic injury of skeletal muscles is generally observed in the clinical field, such as
45 intramuscular injection, and is characterized by muscle regeneration after immune cell
46 infiltration. Therefore, autoimmune diseases could affect the repair process of traumatic
47 skeletal muscle injury, but their pathological modification has been unclear. This study
48 revealed the histopathological features of abdominal walls that received a needle puncture in
49 an autoimmune disease mouse model. Infiltrations of lymphocytes, particularly T cells,
50 around the injured muscle were substantially increased in mice with autoimmune disease.
51 However, these muscles were regenerated without any abnormalities, such as fibrosis, with
52 proliferation of fibro-adipogenic progenitors, in such mild injury. Therefore, this study
53 provided the basic knowledge to understand the process and features of muscle regeneration
54 in case of mild traumatic injuries in patients with autoimmune disease.

55

56 **Introduction**

57 Skeletal muscles continue regeneration and reconstruction to maintain their morpho-function
58 during the lifetime. In a healthy status, skeletal muscles contain a pool of satellite cells, which
59 are undifferentiated and quiescent mononuclear cells surrounding mature skeletal muscle
60 cells. Their activation and differentiation are crucial for skeletal muscle regeneration
61 accompanied by infiltrations of immune cells and activation of fibro-adipogenic progenitors
62 (FAPs). FAPs are present in the skeletal muscle stroma, regulate immune cell accumulation
63 and function, and influence the homeostasis of the satellite cell pool and myogenesis.¹ FAPs
64 are not myogenic cells but have the bipotential abilities to differentiate into fibroblasts or
65 adipogenic cells, and their cell fate is strongly affected by the external environment in the
66 tissues.^{2,3}

67 In a familiar environment, several skeletal muscle injuries are caused by accidents or
68 medical treatments, such as surgery or intramuscular injection (i.m.). Generally, at the acute
69 phase of traumatic skeletal muscle injury, myocytes undergo necrosis, and immune cells
70 accumulate in response to skeletal muscle injury during the repair process. Neutrophils start to
71 accumulate starting from 24 h after injury, followed by macrophage infiltration and satellite
72 cell proliferation. From the data of a mouse model for severe traumatic skeletal muscle
73 injury⁴, C57BL/6 mice (B6) showed a predominant accumulation of CD11b-positive
74 (CD11b⁺) cells at 3 days after injury induced by cardiotoxin (CTX) injection into the anterior
75 tibial and quadriceps muscles. In this model, CD3⁺ T cells increased at the injury site, and
76 their numbers were maintained until day 5 after injection. At that time point, macrophage
77 infiltrations switched from an inflammatory M1 to an anti-inflammatory M2 phenotype, and
78 satellite cell proliferation reached its peak. Then, immune cells decreased whereas satellite
79 cells showed self-duplication or formed centronuclear myotubes. Thus, the injured skeletal
80 muscles were repaired by collaboration with the immune system.

81 On the other hand, several factors, such as genetic abnormalities, systemic diseases, and
82 aging, cause the abnormalities of remodeling or regeneration, such as fibrosis, ossification, or
83 adipogenesis in the skeletal muscle. For example, Schwartz-Jampel syndrome and muscular

84 dystrophy are associated with genetic traits and cause skeletal muscle degeneration and
85 abnormal regeneration.⁵ Moreover, in traumatic skeletal muscle injury, in addition to a
86 persistent infiltration of inflammatory cells, FAPs may proliferate and induce fibrosis,⁶ even
87 though they would normally trigger apoptosis. Therefore, traumatic injuries by medical
88 treatments also induce the abnormalities of remodeling or regeneration in skeletal muscles. In
89 medical settings, advances in acute care and traumatic surgery have increased the
90 opportunities for medically induced traumatic skeletal muscle injuries. For example, surgical
91 trauma to the jaw, such as bone resection, intramuscular injection, dental treatment, or
92 surgery, is the most common cause of heterotopic ossification of the masticatory muscles and
93 may cause deformity of the jaw joint.⁷ Autoimmune diseases (AIDs) are characterized by the
94 production of autoantibodies or autoreactive cells in response to tissues. The number of
95 patients with AIDs is increasing each year.⁸ Because skeletal muscle regeneration is related to
96 the immune system, their abnormalities might be closely associated. Idiopathic myositis is an
97 AID that targets skeletal muscle and is characterized by weakness, inflammation, and fibrosis
98 of skeletal muscles.⁹ On the other hand, the pathological correlation between autoimmune
99 abnormalities and traumatic skeletal muscle injury remains unclear.

100 The pathological crosstalk between the immune system and skeletal muscle was mainly
101 examined by using severe experimental models induced by i.m. of myopathic reagents, such
102 as CTX;¹⁰ however, few studies have examined this crosstalk in milder skeletal muscle injury.
103 In this study, we focused on needle puncture as a traumatic skeletal muscle injury and
104 investigated the relationship between skeletal muscle remodeling or regeneration and
105 autoimmune abnormalities by using a mouse model. The results obtained from the present
106 study revealed the effects of mild traumatic skeletal muscle injury on mice with healthy or
107 autoimmune abnormalities. Therefore, this study provides crucial insight into evaluation of
108 the effects of universally practiced medical treatments, particularly skeletal muscle needle
109 puncture.

110 **Materials and methods**

111 *Animals*

112 Male MRL/MpJ (MpJ), MRL/MpJ-*Fas*^{lpr/lpr} (lpr), and B6 mice were purchased from Japan
113 SLC, Inc. (Hamamatsu, Japan) and maintained under specific pathogen-free conditions.
114 Animal experimentation was approved by the Institutional Animal Care and Use Committee
115 of the Faculty of Veterinary Medicine, Hokkaido University (approval No. 21-0008). All
116 experimental animals were handled by the Guide for the Care and Use of Laboratory Animals,
117 Faculty of Veterinary Medicine, Hokkaido University (approved by the Association for
118 Assessment and Accreditation of Laboratory Animal Care International).

119

120 *Preparation of the abdominal wall puncture model*

121 At 6–7 months of age, mice were weighed and anesthetized by intraperitoneal injection (i.p.)
122 of a mixture of medetomidine (0.3 mg/kg), midazolam (4 mg/kg), and butorphanol (5 mg/kg)
123 from the left area of the abdominal wall. Then, the abdominal skin was shaved and
124 disinfected, and mice were gently kept warm and recovered by administering atipamezole (0.3
125 mg/kg, i.p.) with a 21G needle from the right-side abdominal wall. Then this area was
126 recorded as a needle puncture site. Mice were euthanized 1, 3 or 8 days after the needle
127 puncture, and tissue samples were collected (Figure 1A). All mice were administered
128 bromodeoxyuridine (BrdU) (1 mg/10 g body weight (BW), i.p.) 2-3h before they euthanized.
129 Euthanasia was performed by cutting the femoral artery and dislocating the cervical vertebrae
130 under deep anesthesia. The abdominal wall, including the external and internal oblique
131 abdominal muscle and the transverse abdominis muscle, was collected without distinction
132 because they were difficult to separately isolate. The right part of abdominal wall was used as
133 the needle puncture site. Subsequently, the spleens were immediately collected and weighed.

134

135 *Serological analysis*

136 For an index of AID development, serum levels of anti-double-stranded DNA (dsDNA)
137 antibodies were measured to evaluate systemic autoimmune conditions using LBIS anti

138 dsDNA-mouse ELISA kit (FUJIFILM Wako Pure Chemical Corporation; Osaka, Japan)
139 according to manufacturer's instructions. As another index, the ratio of the spleen weight
140 (SPW) to BW (SPW/BW) was calculated.

141

142 *Histological analysis*

143 Needle puncture was performed perpendicular to the abdominal wall muscle, and the entire
144 abdominal wall, including the punctured area, was subsequently collected. The abdominal
145 wall was then stretched onto a flat surface, fixed in 4% paraformaldehyde overnight at 4 °C,
146 and embedded in paraffin. Longitudinal sections (3–4- μ m thick) were cut through the
147 abdominal wall muscle; each section therefore contained a transverse cross-section of the
148 punctured area. Deparaffinized sections were stained with hematoxylin-eosin (HE) or Elastica
149 van Gieson (EVG), and The punctured area was identified by observing injured muscles.

150

151 *Immunohistochemistry (IHC) and immunofluorescence (IF)*

152 IHC for IBA1, Gr-1, B220, CD3, and BrdU was performed to detect the macrophages,
153 neutrophils, B cells, T cells, and proliferating cells, respectively. Briefly, paraffin sections
154 were deparaffinized, and antigen was retrieved by heating. Then, to block internal peroxidase
155 activity, the sections were soaked in methanol containing 0.3% H₂O₂ for 20 min at 25 °C.
156 After washing 3 times in phosphate-buffered saline (PBS), the sections were incubated with a
157 blocking serum for 1 h at room temperature to block non-specific reactions. Then, sections
158 were incubated with primary antibodies overnight at 4 °C. After washing 3 times in PBS, the
159 sections were incubated with secondary antibodies for 30 min at 25 °C and washed 3 times in
160 PBS. Consequently, the sections were incubated with streptavidin-conjugated horseradish
161 peroxidase (SABPO® kit, Nichirei; Tokyo, Japan) for 30 min at 25 °C, washed 3 times in
162 PBS, and the immunopositive reaction was visualized with 10 mg of 3,3'-diaminobenzidine
163 tetrahydrochloride in 50 mL 0.05 M Tris-H₂O₂ solution. Finally, the sections were
164 counterstained with hematoxylin. The details of the antibodies, antigen retrieval, and blocking
165 are listed in Table 1.

166 IF was performed to detect BrdU-incorporating cells and positive cells for paired box 7
167 (PAX7) or platelet-derived growth factor receptor α (PDGFR α). The paraffin sections were
168 deparaffinized, subjected to antigen retrieval, and then blocked using normal donkey serum,
169 using the same protocol as that for IHC. After overnight incubation with the primary
170 antibodies, sections were incubated with the secondary antibody for 1 h at 25 °C. The sections
171 were sealed with a water-soluble encapsulant and observed under fluorescence microscopy
172 (BZX-710; Keyence; Osaka, Japan). The details of antigen retrieval, dilution, and source of
173 antibodies are listed in Table 1.

174

175 *Histoplanimetry*

176 IHC sections were converted to virtual slides by Nano Zoomer 2.0 RS (Hamamatsu Photonics
177 Co., Ltd.; Hamamatsu, Japan). Using NDP view2 (Hamamatsu Photonics Co., Ltd.), 3–5
178 images of the puncture site were randomly selected at 40 \times magnification. Then, the number of
179 total and positive cell nuclei in necrotic areas was manually identified in the IHC-stained
180 images of IBA1, Gr-1, B220, CD3, and BrdU. Each percentage of the number of positive cell
181 nuclei to total cell nuclei in the injury region was calculated. Because of the strong
182 nonspecific response of IBA1 to necrotic cytoplasm, BZ-X710 and BZ-H3C (Keyence) color
183 discrimination was used to identify positive cells.

184

185 *Statistical analysis*

186 The results were expressed as the mean \pm standard error (SE) and analyzed by non-parametric
187 statistical methods. Significant difference between 2 groups was assessed by Mann-Whitney
188 *U* test. Kruskal-Wallis test was used to compare the numerical results, and multiple
189 comparisons were performed using Dunnett's test when significant differences were observed.
190 The correlation was analyzed using Spearman's correlation test. A *P* value lower than 0.05
191 was regarded as a significant difference in all analyses. **All statistical analysis was performed**
192 **using IBM SPSS ver. 28.0.1 (IBM Japan Ltd., Tokyo, Japan)**

193 **Results**

194 ***Indices of AID***

195 According to Figure 1A, treatment and sampling were performed at 1, 3, and 8 days. Serum
196 levels of anti-dsDNA antibody and the ratio of SPW/BW of 6–7-month-old MpJ and lpr mice
197 were measured at each time point as indices of AID (Figures 1B and C). Lpr mice showed
198 significantly higher values compared with those of MpJ mice in both indices at all time points
199 (note logarithmic display). Furthermore, MpJ mice at 8 days and lpr mice at 3 days showed
200 significantly higher serum levels of anti-dsDNA antibody compared to each value at 1 day.
201 These data indicated that the lpr mice used in this experiment developed a systemic AID.

202

203 ***Histological alterations of the puncture region over time***

204 As shown in the images of HE-stained section from MpJ at 1 day (Figure 2A), the injured
205 myofibers at the puncture region showed necrosis and a paler color compared with that of the
206 surrounding intact myofibers. These alternations were observed along with the longitudinal
207 axis of injured myofibers and separated the injured and healthy skeletal muscle tissue.
208 Therefore, these necrotic areas were defined as the injured area surrounded by healthy
209 myofibers.

210 Figure 2B shows the HE-stained cross sections of the abdominal wall muscle, including
211 the injured area, and summarizes the histological alternations of MpJ and lpr mice during the
212 observation period. Briefly, MpJ and lpr mice showed similar histological alterations. On day
213 1, the injured myofibers were necrotic with a few scattered infiltrations of inflammatory cells
214 and blood cells. On day 3, abundant mononuclear cell infiltrations were observed between the
215 necrotic myofibers, indicating induction of local inflammatory response. On day 8, the
216 number of infiltrating cells decreased, and many regenerative myotubes with central nuclei
217 were observed. Thus, comparable histological changes in the injury and regenerative process
218 of skeletal muscles by needle puncture were observed, and the features of inflammatory
219 response at 3 days and regeneration at 8 days were prominent in both strains.

220

221 ***Inflammatory cell infiltration at the puncture region***

222 Next, we assessed macrophages and neutrophils, which are known to be initially induced in
223 the region of skeletal muscle injury.¹¹ Moreover, B and T cells were examined to evaluate
224 their relationship with AIDs.

225 As shown in Figure 3, IBA1⁺ macrophages were abundantly observed at 3 days among
226 all examined inflammatory cells in both strains and tended to be comparable or decreased at 8
227 days (Figure 3A). On the other hand, common to both strains, abundant Gr-1⁺ neutrophils
228 were observed at 1 day and decreased onward (Figure 3B). Quantification of these
229 inflammatory cells showed that IBA1⁺ macrophages exhibited the highest values at 3 days in
230 both strains without strain- or time course-related significant differences (Figure 3C). For Gr-
231 1⁺ neutrophils, the quantitative values at 1 day were significantly higher than those at other
232 time points in both strains without strain differences (Figure 3D).

233 As shown in Figures 4A and B, the number of lymphocytes was lower than that of
234 macrophages and neutrophils, but some differences were detected between studied strains.
235 Briefly, the quantification of these cells showed no change in B220⁺ B cells of MpJ mice
236 during the observation periods, but *lpr* mice showed the highest values at 1 day with a higher
237 tendency compared with that of MpJ ($P=0.063$) and then decreased (Figure 4C). Similar
238 tendency was also observed in CD3⁺ T cells, and *lpr* mice showed a significantly higher value
239 compared with that of MpJ at 1 day and then decreased (Figure 4D). Thus, *lpr* mice were
240 suggested to induce more lymphocyte infiltration among injured myofibers at 1 day compared
241 with that of MpJ mice.

242

243 ***Proliferating cells at the puncture region***

244 During skeletal muscle regeneration, the formation of myotubes by proliferation,
245 differentiation, and fusion of satellite cells is important.¹²

246 Figure 5A shows the IHC images of BrdU-incorporating proliferating cells in the
247 puncture region of MpJ and *lpr* mice. Few positive cells were detected at 1 day in both strains,
248 but they were observed as numerous at 3 days and tended to decrease at 8 days.

249 Quantification of BrdU-incorporating cells (Figure 5B) showed that the values in MpJ were
250 significantly higher at 3 days compared with those at 1 and 8 days. Lpr mice also showed a
251 similar tendency, and a significant difference was observed between 1 and 3 days. Further, the
252 values at 3 days tended to be higher in MpJ than those in lpr mice ($P=0.095$).

253 Next, the cell type of BrdU-incorporating cells was investigated by assessment of IF.
254 PAX7 and PDGFR α , which were used as markers for satellite cell activation from quiescent
255 to proliferative stages and for FAPs, which are mesenchymal progenitor cells known to
256 influence myogenesis by regulating immune cell accumulation and function and by providing
257 extracellular matrix components.^{2,13} In both strains, almost all PAX7⁺ nuclei showed the
258 merged reactions with BrdU-incorporating nuclei during the observation period (Figures 5C).
259 On the other hand, PDGFR α ⁺ cells rarely showed BrdU incorporation (Figures 5C). This
260 suggests that many satellite cells have been activated from the quiescent to the proliferative
261 phase at the 3 days injury region.

262 To confirm the influence of the genetic background of the mice, the same experiment
263 was conducted with B6 mice (Supplemental figure 1). We found that all BrdU-incorporating
264 nuclei observed were PAX7⁺ nuclei at 3 days, similar to MpJ and lpr mice.

265

266 *Verification of fibrosis as a regenerative abnormality*

267 Finally, to examine the fibrosis associated with the skeletal muscle injury, EVG-stained
268 sections were observed. Figure 6 shows EVG-stained cross sections of the abdominal wall
269 muscle, including the injured area, and summarizes the histological alterations over time in
270 MpJ and lpr mice. At 1 day, it showed a decrease in the staining intensity of picric acid⁺
271 myofibers at the injury region owing to necrosis, and a very small amount of collagen fibers
272 were observed in the interstitium of the skeletal muscles. At 3 days, there was a notable
273 increase in collagen fibers, and the necrotic tissue showed abundant cells, including
274 infiltrating cells. However, at 8 days, when skeletal muscle regeneration was initiated,
275 centronuclear myocytes (as shown in Figure 2B) were formed similarly to normal myofibers,
276 and a significant decrease in collagen fibers was observed, confirming a smooth regeneration

277 process. Thus, in both strains, no fibrotic features were detected in the injury region,
278 indicating normal regeneration of skeletal muscles.

279

280

281 **Discussion**

282 This study aimed to clarify the effects of systemic immunological abnormalities on the repair
283 processes of injured skeletal muscles. In particular, the authors focused on traumatic skeletal
284 muscle injuries that are universally caused by medical treatments, such as i.m. The skeletal
285 muscles of abdominal walls in AID-prone *lpr* mice were punctured. Their histological
286 alterations with immunological indices at several time points were compared with those of
287 MpJ, a healthy control strain of *lpr* mice. Although both strains showed similar skeletal
288 muscle injuries and regeneration without fibrotic features, several features, such as
289 lymphocyte infiltration dynamics, were different in these processes.

290 Several studies using mouse models with CTX- or glycerol-induced muscle injuries
291 indicated that satellite cell activation continued up to day 4 after injury, myotubes with central
292 nuclei were evident at day 7, and the diameter of these myotubes continued to increase until
293 day 14.¹⁴ In particular, the CTX model is suitable for studying uniform and complete
294 regeneration. Fibrosis is mild and transient, and the CTX model is used globally. On the other
295 hand, experimental glycerol myopathy could be a suitable model to study the pathophysiology
296 of Duchenne muscular dystrophy, where impaired regeneration is observed, and fibrosis
297 occurs. In the present study, we focused on needle puncture as a traumatic skeletal muscle
298 injury and investigated the relationship between the remodeling or regeneration of skeletal
299 muscle and autoimmune abnormalities by using a mouse model; therefore, we compared the
300 result from CTX-model with our obtained data.

301 In the present study, sampling points were scheduled at day 1 (immediately after
302 injury/acute inflammation), day 3 (active inflammation), and day 8 (regeneration) to elucidate
303 the effects of AID on muscle injury inflammation and regeneration, with a particular focus on
304 the cells localized to puncture lesions. Although fibrosis starts at day 4 due to the proliferation
305 of fibroblasts in more severe cases,¹⁴ no significant collagen deposition was noted in our
306 model on day 3 and 8 after puncture. Furthermore, on day 8, there was no significant
307 difference in the regeneration features (see Figures 2, 5, 6). Therefore, we concluded that
308 fibrosis, as indicated by the proliferations of PDGFR α ⁺ cells, did not occur in both healthy

309 and AID groups in this puncture model.

310 For examined indices of autoimmune abnormalities, the serum level of anti-dsDNA antibody
311 was gradually and significantly increased in MpJ mice from 1 to 8 days, and this increase was
312 more prominent in lpr mice from 1 to 3 days. For supportive data, correlation analysis was
313 performed between indices of AID and the quantitative values of examined cells (Supplemental
314 table 1). Although no significant correlation was observed within each group, the numbers of
315 CD3⁺ T cells and B220⁺ B cells temporarily increased at 1 and 3 days, respectively, when the
316 serum level of the anti-dsDNA antibody increased. This result was observed using all groups.
317 Myositis autoantigens, such as histidyl tRNA synthetase, Mi-2, U1-70kD, Ku/the catalytic
318 subunit of DNA-dependent protein kinase, are expressed at high levels in myositis skeletal
319 muscles, whereas they are very low in normal skeletal muscles; their expressions are
320 increased in cells that have features of regenerating skeletal muscle cells.¹⁵ Furthermore,
321 traumatic skeletal muscle injury stimulates the production of autoantibodies following
322 inflammation.¹⁶ Therefore, the authors supposed that lpr mice also developed myositis with
323 the increase of serum autoantibodies, such as an anti-dsDNA antibody, by the increase of this
324 autoantigen expression after skeletal muscle injuries. However, the myositis feature was not
325 clearly observed in lpr mice, and the skeletal muscle inflammation was mild and limited in a
326 locally injured area and eventually diminished at 8 days. Therefore, AID-prone mice induced
327 the production of anti-dsDNA antibodies more abundantly compared with that in healthy ones
328 after skeletal muscle injuries although they did not develop myositis. Furthermore, the authors
329 assumed that the pathological effects on systemic organs by autoantibody increase might be
330 mild after local traumatic skeletal muscle injury because no significant alteration was detected
331 in the ratio of SPW/BW from 1 to 3 days in both strains.

332 Traumatic skeletal muscle injuries in both strains resulted in infiltration of immune cells
333 that contribute to innate immune responses, such as neutrophils and macrophages. However, it
334 was characterized by a more abundant infiltration of lymphocytes, especially T cells, in lpr
335 mice. T cells (CD45⁺ CD11b⁻ CD3⁺ cells) infiltrate the skeletal muscle after injury, and their
336 number in the skeletal muscle peaked at 3 days and remained constant through 5 days before

337 declining at 7 days by one CTX injection to the anterior tibialis and quadriceps muscles of 8–
338 10-week-old female B6 mice. Furthermore, it was suggested that they activated T helper cells,
339 consistently expressing CD4 but not CD8 marker.⁴ Importantly, *lpr* mice developed
340 progressive lymphadenopathy after 6 weeks of age owing to accumulation of an abnormal
341 population of CD4⁻ CD8⁻ double negative T cells.^{17,18} It is unclear whether these lymphocytes
342 in *lpr* mice recognized specific antigens derived from the destroyed myocytes or this was the
343 result of a nonspecific immune response; however, the *lpr* mutation itself and/or the
344 associated autoimmune abnormalities would affect the infiltration of lymphocytes, especially
345 T cells, at the injured skeletal muscle area.

346 Many myogenic progenitor cells and FAPs were detected as BrdU-incorporating cells at
347 3–4 days during the regeneration process after extensive skeletal muscle disruption with CTX
348 i.m. to the hindlimbs of B6 mice.³ FAPs are quiescent in intact skeletal muscle but proliferate
349 in response to skeletal muscle injury to stimulate activation and differentiation of satellite
350 cells.^{3,19,20} Therefore, in this study, PAX7⁺ satellite cells and PDGFR α ⁺ FAPs were co-stained,
351 but only the formers proliferated at the injury site after puncture in both groups. Considering
352 the influence of the genetic background of the mice on this phenotype, the same experiments
353 were conducted with B6 mice (Supplemental figure 1); the results showed that, similar to MpJ
354 and *lpr* mice, all BrdU-incorporating cells were satellite cells, and FAPs did not show
355 incorporation. Therefore, it was concluded that FAPs activation is not induced in skeletal
356 muscle injury by puncture, although satellite cells locally proliferate. Regarding the
357 differences from previous studies using the CTX i.m. model,^{3,19,20} the variation in the degree
358 of skeletal muscle injury, rather than the genetic background of the mice, was considered to
359 affect the proliferation kinetics of satellite cells and FAPs.

360 In healthy skeletal muscle tissue, satellite cells go from a quiescent state to a
361 proliferative phase a few days after injury and simultaneously start to repair the injured and
362 necrotic sites.^{3,21} Increased proliferating cells at the injury site at 3 days tended to be less in
363 *lpr* than those in MpJ mice. Importantly, the autoantibody concentration of healthy MpJ mice
364 progressively increased up to 8 days. Because the majorities of the proliferating cells are

365 satellite cells, the change in MpJ autoantibody concentration may have reflected a transient
366 increase in regenerating myocytes expressing autoreactive autoantigen.^{14,22} On the other hand,
367 at 3 days, lpr tended to have the same or fewer proliferating cells as those in MpJ mice, and
368 correlation analysis of all mice showed a negative correlation between the index of AID and
369 the number of proliferating cells. However, the increased autoantibody concentration at 3 days
370 was more remarkable in lpr than that in MpJ mice, suggesting that this phenotype reflects
371 excessive activation of the immune system to recognize the autoantigen rather than the
372 amount of autoantigen derived from the regenerating myocytes.²³ In addition, satellite cells
373 and FAPs in healthy skeletal muscle tissue distant from the injury site were compared
374 between MpJ and lpr mice, but no difference was detected in their number or localization
375 (data not shown).

376 Both strains had no remarkable fibrosis findings at the injury site. Essentially, fibrosis
377 occurs in damaged skeletal muscle when persistent inflammatory cell infiltration and fibroblast
378 activation produce a mass of nonfunctional fibrous tissue.^{24,25} Apoptosis of FAPs is required for
379 normal skeletal muscle regeneration and the failure of apoptosis leads to skeletal muscle fibrosis
380 by increasing the survival rate of FAPs.^{19,20} On the other hand, in this study, neither proliferation
381 of FAPs nor fibrosis were detected. Thus, it can be assumed that AIDs affect lymphocyte
382 induction and satellite cell dynamics in the early stages of injury, whereas minor skeletal muscle
383 injuries, such as puncture, do not directly lead to fibrosis or other regenerative abnormalities.

384 In conclusion, this study demonstrated that autoimmune abnormalities modified the
385 processes of inflammation and repair after skeletal muscle injury, whereas the skeletal
386 muscles injured by mild trauma, such as needle puncture can be normally regenerated. This
387 might suggest that the induction of immune-associated abnormal skeletal muscle regeneration
388 is affected by the kinds of reagents or adjuvants rather than the puncture itself, whereas the
389 accumulation of data from various conditions and diseases would be crucial. The obtained
390 results also emphasized an important perspective for future studies, especially focusing on the
391 significance of less invasive medical treatment for patients with autoimmune abnormalities.

392

393 **Limitations**

394 This study did not address the long-term effect of muscle puncture on AID status. Furthermore,
395 a future study into how muscle-derived autoantigens influence systemic AID phenotypes would
396 help comprehensively elucidate the effect of muscle puncture on AID and vice versa (e.g.,
397 muscle puncture before AID development at a young age, and analysis at 6–8 months of age
398 using MRL/lpr) with functional effects, such as muscle contractility.

399 **Authors' contributions**

400 MM, OI, and YK designed and performed the experiments. MM, YO, and TN provided the
401 samples and analyzed the data. All authors were involved in writing the paper and have
402 approved the final manuscript.

403

404 **Declaration of conflicting interests**

405 The authors declared no potential conflicts of interest with respect to the research, authorship,
406 and/or publication of this article.

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411 **Figure legends**

412 **Figure 1. Experimental schedule and indices for autoimmune abnormalities. (A)**

413 Experimental schedule. (B) The serum levels of anti-double-stranded DNA (dsDNA)
414 antibody. (C) The percentage of spleen weight (SPW) to body weight (BW). The number of
415 samples used is as follows: n=5 (MpJ/1 day), 4 (MpJ/3 days), 5 (MpJ/8 days), 4 (lpr/1 day), 4
416 (lpr/3 days), 4 (lpr/8 days). Each bar represents the mean \pm SE. *: significant difference from
417 the other strain at the same days that elapsed since puncture (Mann-Whitney *U*-test, * $P < 0.05$).
418 #: significant difference from the same strain at other days elapsed since puncture (Kruskal-
419 Wallis test followed by Scheffé's method, # $P < 0.05$). MpJ: MRL/MpJ, lpr: MRL/MpJ-*Fas*^{lpr/lpr}.

420

421 **Figure 2. Histological features of myofibers in the injury region after needle puncture in**

422 **the muscle. (A)** Histological features of the abdominal wall muscle of MpJ mice at 1 day
423 around the puncture region. The blue line represents the boundary between the injury and
424 healthy regions. Myofibers at the injury region have necrotic cytoplasm. Bars=100 μ m. *:
425 **Normal myofibers. (B)** Histological alternations at the injury region of MpJ and lpr mice over
426 time. Both strains show cellular infiltration owing to inflammatory response at 3 days. At 8
427 days, the number of infiltrating cells decreases, and many regenerative myotubes with central
428 nuclei are observed (arrowheads). **Insets indicate the squared area.** Bars=100 μ m.
429 Hematoxylin-eosin staining. MpJ: MRL/MpJ, lpr: MRL/MpJ-*Fas*^{lpr/lpr}.

430

431 **Figure 3. Infiltration of macrophages and neutrophils at the injury region after needle**

432 **puncture in the muscle. (A)** IBA1-positive macrophages (arrowheads). More cells are
433 observed at 3 days in both strains. (B) Gr-1-positive neutrophils (arrowheads). More cells are
434 observed at 1 day in both strains. Immunohistochemistry staining. Bars=100 μ m. The blue
435 line represents the boundary between the injury and healthy regions. (C) The percentage of
436 the number of IBA1-positive cell nuclei to that of all cell nuclei in the injury region. (D) The
437 percentage of the number of Gr-1-positive cell nuclei to that of all cell nuclei in the injury
438 region. ND: Not detected. The number of samples used is as follows: n=5 (MpJ/1 day), 5

439 (MpJ/3 days), 5 (MpJ/8 days), 4 (lpr/1 day), 5 (lpr/3 days), 4 (lpr/8 days). Each bar represents
440 the mean \pm SE. 3d, 8d: significant difference from the same strain at 3 days and 8 days since
441 puncture (Kruskal-Wallis test followed by Scheffé's method, $^{\#}P < 0.05$). MpJ: MRL/MpJ, lpr:
442 MRL/MpJ-*Fas*^{lpr/lpr}.

443

444 **Figure 4. Infiltration of lymphocytes at the injury region after needle puncture in the**
445 **muscle.** (A) B220-positive B cells (arrowheads). Slightly more cells are observed in lpr at 1
446 day. (B) CD3-positive T cells (arrowheads). More cells are observed in lpr at 1 day.
447 Immunohistochemistry staining. Bars=100 μ m. The blue line represents the boundary
448 between the injury and healthy regions. (C) The percentage of the number of B220-positive
449 cell nuclei to that of all cell nuclei in the injury region. (D) The percentage of the number of
450 CD3-positive cell nuclei to that of all cell nuclei in the injury region. The number of samples
451 used is as follows: n=5 (MpJ/1 day), 5 (MpJ/3 days), 5 (MpJ/8 days), 4 (lpr/1 day), 5 (lpr/3
452 days), 4 (lpr/8 days). Each bar represents the mean \pm SE. *: significant difference from the
453 other strain at same days elapsed since puncture (Mann-Whitney *U*-test, $*P < 0.05$). MpJ:
454 MRL/MpJ, lpr: MRL/MpJ-*Fas*^{lpr/lpr}.

455

456 **Figure 5. Proliferating cells in the injury region after needle puncture in the muscle.** (A)
457 Bromodeoxyuridine (BrdU)-incorporating cells (arrowhead). The blue line represents the
458 boundary between the injury and healthy regions. More cells are observed at 3 days in both
459 strains. Immunohistochemistry staining. Bars=100 μ m. (B) The percentage of the number of
460 BrdU-incorporating cell nuclei to that of all cell nuclei in the injury region. The number of
461 samples used is as follows: n=5 (MpJ/1 day), 5 (MpJ/3 days), 5 (MpJ/8 days), 4 (lpr/1 day), 5
462 (lpr/3 days), 4 (lpr/8 days). Each bar represents the mean \pm SE. 1d, 1d', 8d': significant
463 difference from the same strain at other days elapsed since puncture (Kruskal-Wallis test
464 followed by Scheffé's method, 1d $P < 0.05$, 1d' 8d' $P < 0.01$). Significant difference from the
465 other strain at same days are analyzed by Mann-Whitney *U*-test. (C) Double
466 immunofluorescence (IF) for BrdU (white/ proliferating cells) and paired box 7 (PAX7)

467 (red/satellite cells) or platelet-derived growth factor receptor α (PDGFR α) (green/ fibro-
468 adipogenic progenitors) at 3 days, 1 and 8 days. At 3 days, almost all BrdU-incorporating
469 cells are merged with PAX7-positive cells (arrowhead), not PDGFR α -positive cells (arrow) in
470 both strains. Similarly, at 1 and 8 days, both PAX7-positive cells and PDGFR α -positive cells
471 are localized at the injury region, and BrdU-incorporating cells merged with only PAX7 in
472 both strains. Blue: Hoechst (nuclei). Bars=100 μ m. MpJ: MRL/MpJ, lpr: MRL/MpJ-*Fas*^{lpr/lpr}.

473

474 **Figure 6. Histological evaluation of fibrosis in the injury region after needle puncture in**
475 **the muscle.** Fibrosis of the abdominal wall muscle is examined at the injury region of MpJ
476 and lpr mice over time. No fibrotic features are detected in the injury region in both strains.
477 Collagen fibers are stained as purple. Elastica van Gieson staining. *(yellow): Normal
478 myofibers.. Bars=100 μ m. MpJ: MRL/MpJ, lpr: MRL/MpJ-*Fas*^{lpr/lpr}.

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- 551

Figure 1

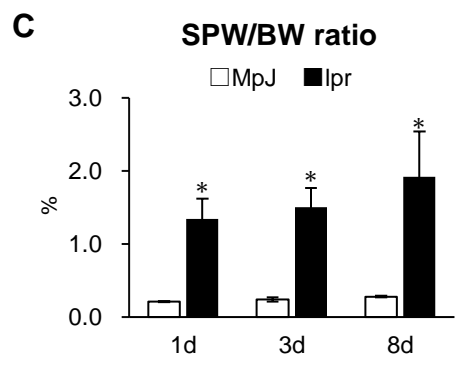
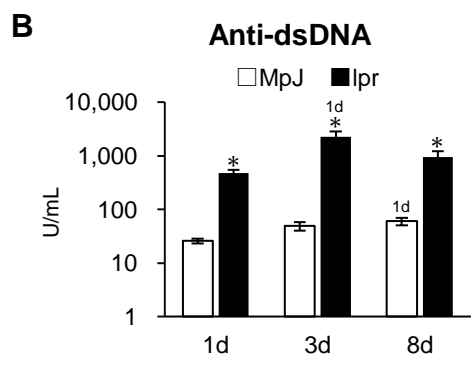
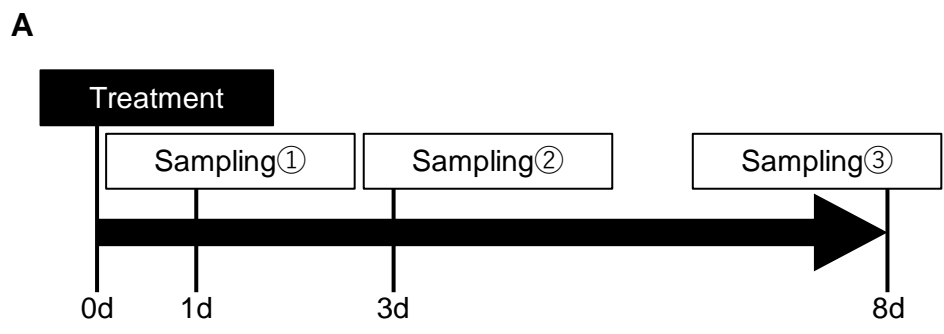


Figure 2

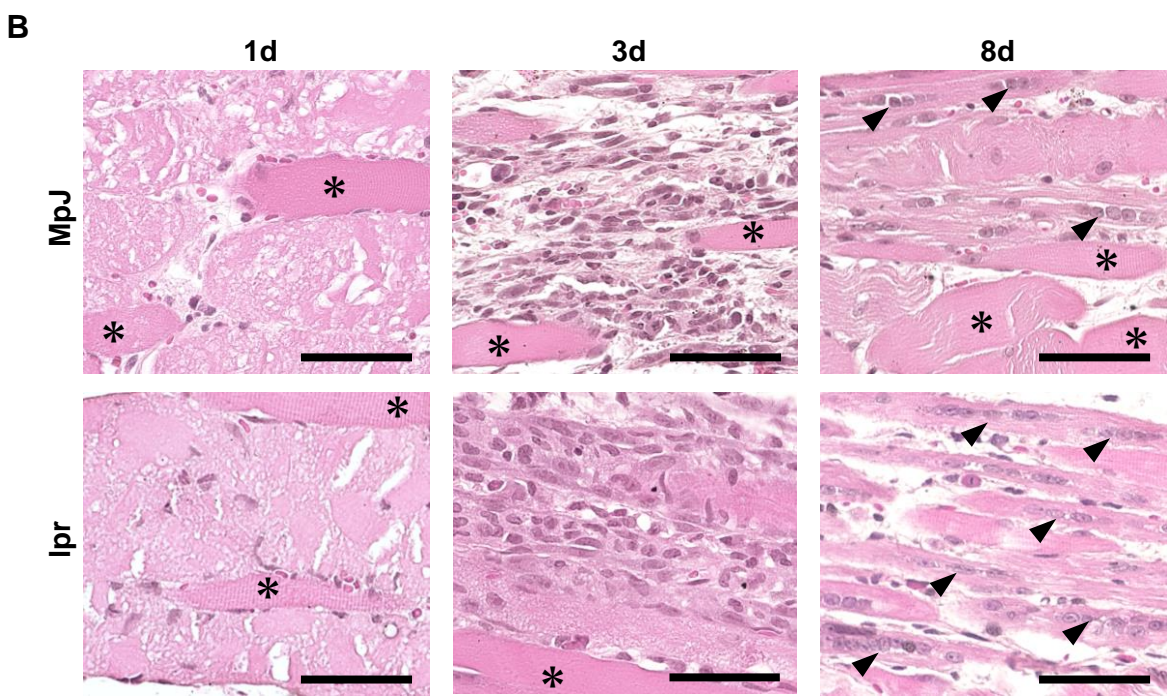
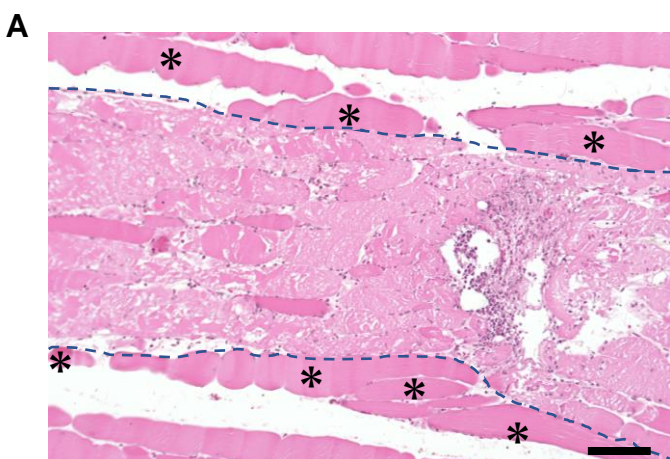


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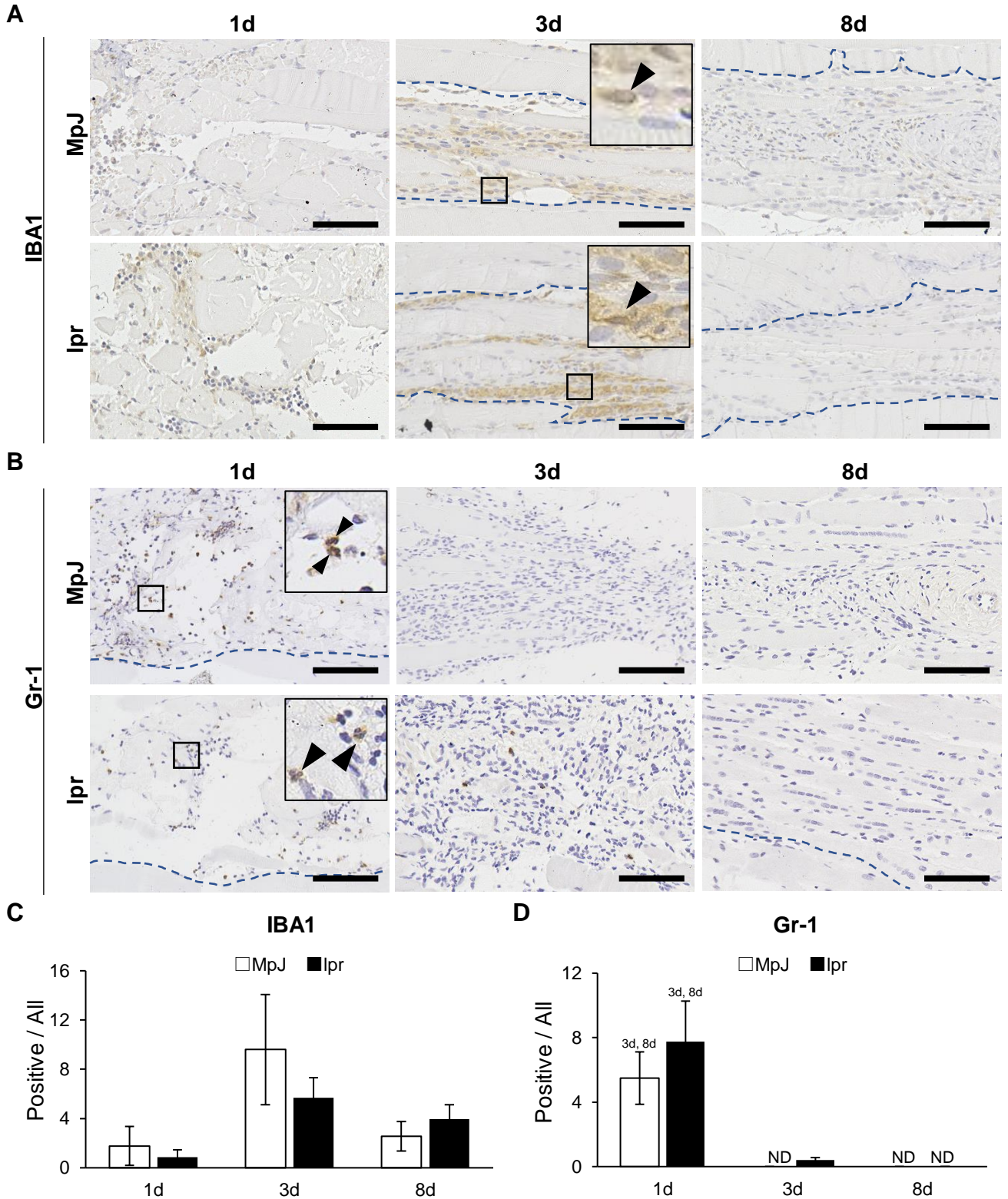


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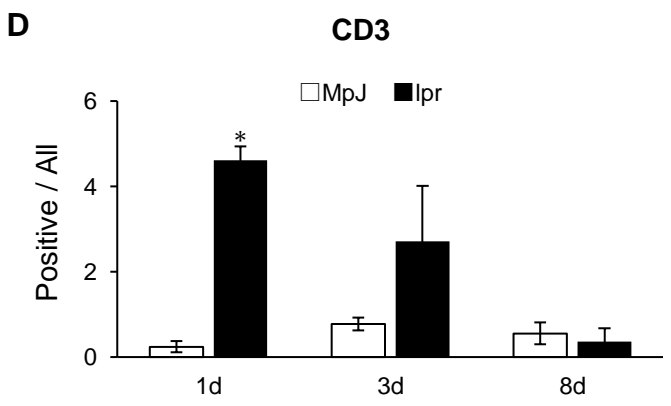
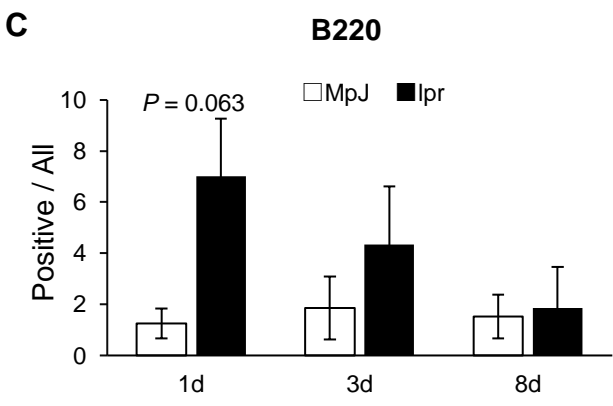
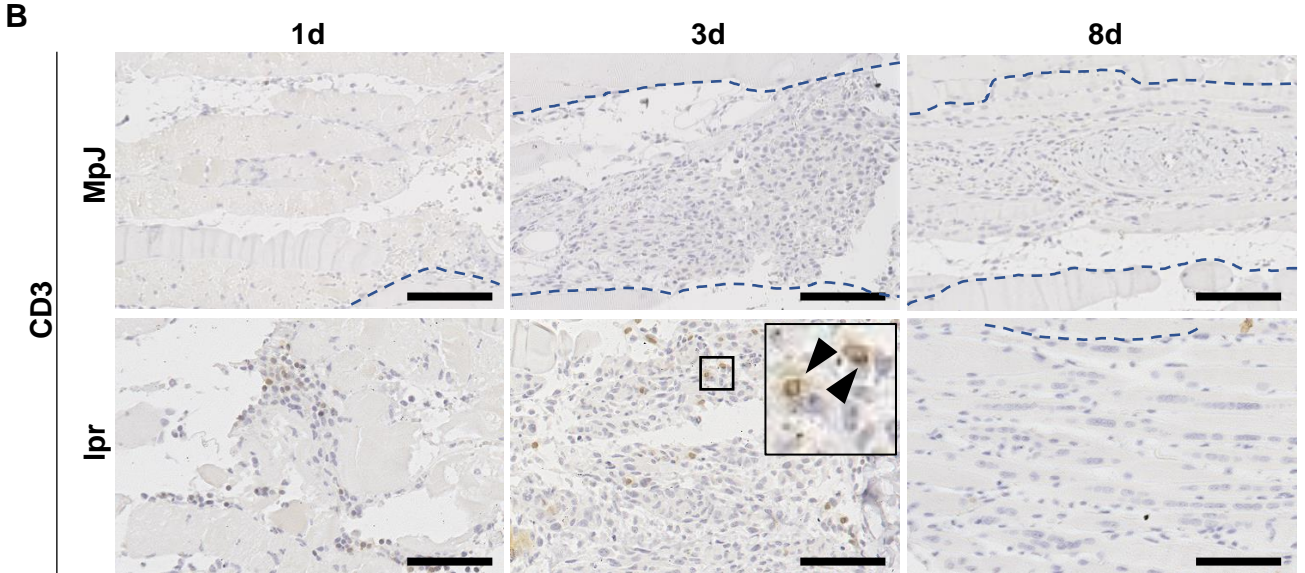
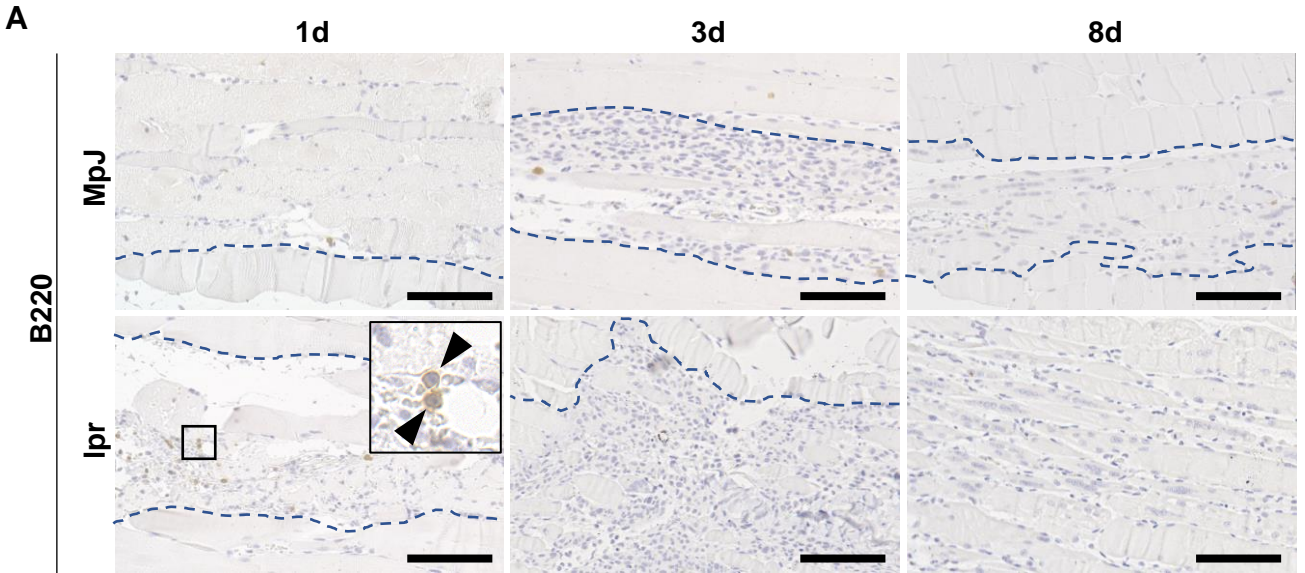


Figure 5

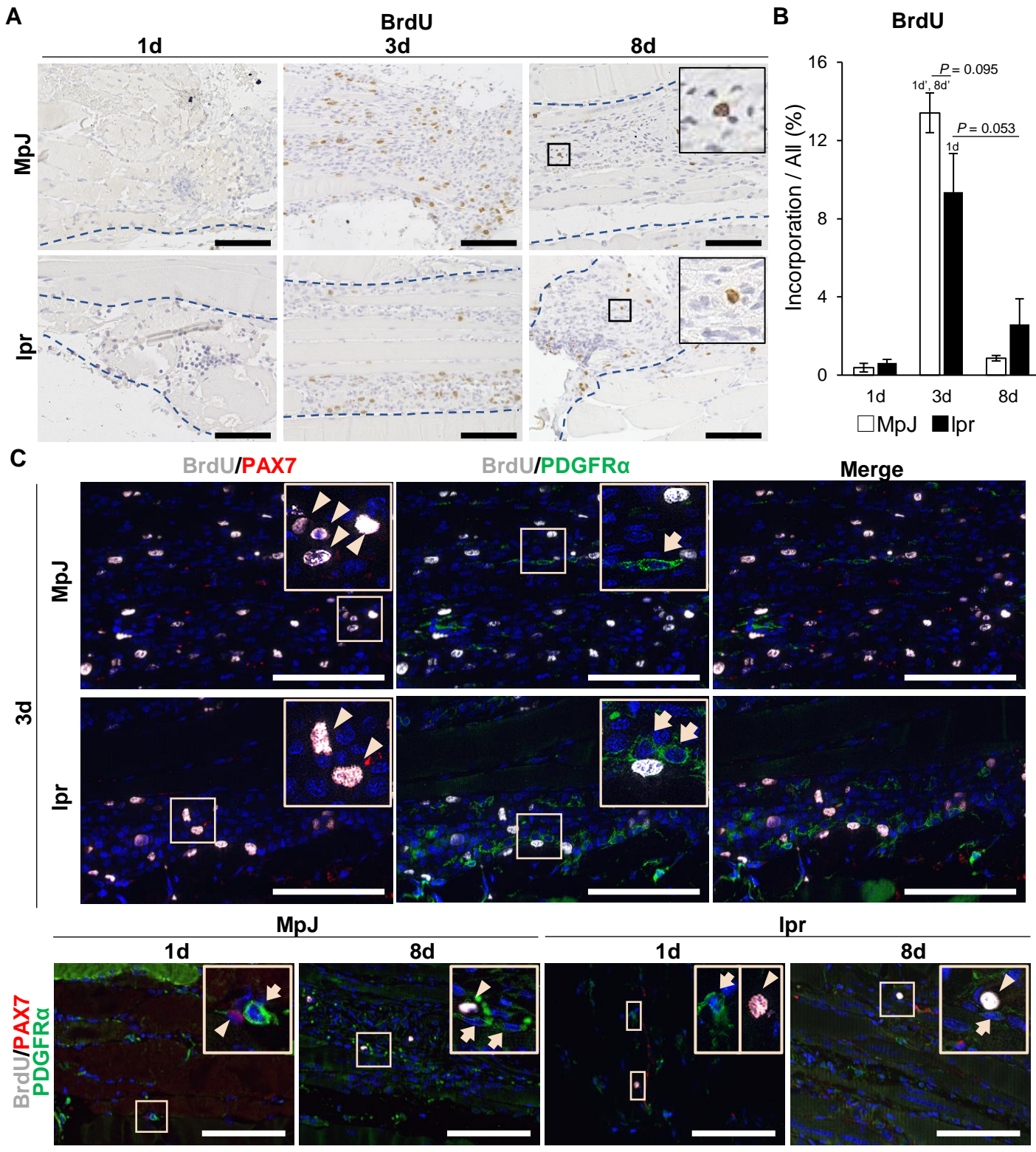
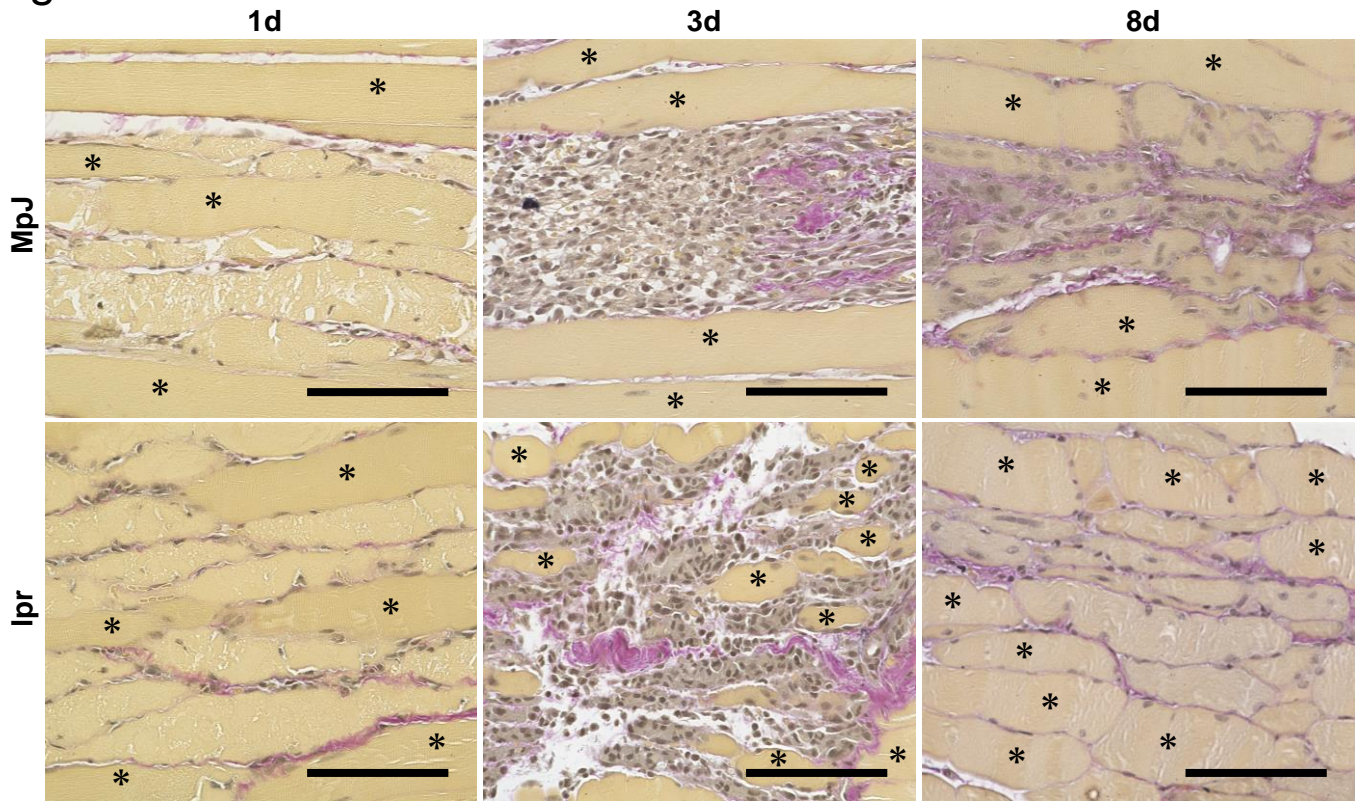


Figure 6



Supplemental figure 1

