

1 **Immunological characterization of a rat model of Duchenne’s disease and**
2 **demonstration of improved muscle strength after anti-CD45RC antibody**
3 **treatment.**

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29

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39 **Competing interests:** IA and CG have registered a patent on the use of anti-CD45RC in
40 Duchenne disease.

41

42 **Abstract.**

43 Duchenne muscular dystrophy (DMD) has as standard pharmacological therapy with
44 corticosteroids (CS) that decrease inflammation and immune responses present in patients
45 and animal models. CS have however limited efficacy and important and numerous side
46 effects. Therefore, there is a need for new anti-inflammatory and pro-tolerogenic treatments
47 that could replace or decrease doses of CS. We first assessed the status of immune system of
48 dystrophin-deficient rats (*Dmd^{mdx}*) that closely reproduce the phenotype of DMD patients.
49 *Dmd^{mdx}* rats showed increased leukocyte infiltration in skeletal and cardiac muscles,
50 containing mostly macrophages but also T cells, and increased expression of several
51 cytokines. Anti-CD45RC Monoclonal antibody (Mab) treatment induced immune tolerance in
52 models of organ transplantation and GVHD (Graft Versus Host Disease). We observed that
53 muscles and blood of DMD patients contained T CD4⁺ and CD8⁺ expressing high levels of
54 CD45RC^{high} cells. Treatment of young *Dmd^{mdx}* rats with anti-CD45RC MAb corrected
55 skeletal muscle strength associated to a depletion of effectors CD45RC^{high} T cells with no
56 obvious side-effects. Prednisolone treatment of *Dmd^{mdx}* rats similarly increased skeletal
57 muscle strength and was also associated to a depletion of effectors CD45RC^{high} cells but
58 resulted in severe weight loss.

59 Overall, *Dmd^{mdx}* rats display important immune inflammatory response and thus represent a
60 useful model to analyze new anti-inflammatory and tolerogenic treatments for DMD. As an
61 example, a new treatment with anti-CD45RC antibodies improved muscle strength in *Dmd^{mdx}*
62 rats as prednisolone did but without side effects. Anti-CD45RC therapy could complement
63 other therapies in DMD patients.

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66

67 **Introduction.**

68 Duchenne muscular dystrophy (DMD) is the most common inherited muscle disease. It is
69 caused by a mutation in the dystrophin gene with a X-chromosomal recessive inheritance that
70 affects 1 of 3,500 male births ¹. It has a severe prognosis with life expectancy ranging from
71 the late teens to the mid-30s. Muscle fibers show necrosis and regeneration/degeneration
72 associated to inflammation with progressive replacement by connective and adipose tissue ¹.

73 The mdx mouse carries a mutation in the *Dmd* gene and is a well-established mouse model of
74 DMD. Nevertheless, the muscle impairment is rather mild in *mdx* mice compared with DMD
75 patients indicating that new animal models are required ².

76 We previously generated *Dmd*-deficient (*Dmd*^{mdx}) rats using TALENs ³. Forelimb and
77 hindlimb muscular strength and spontaneous activity were decreased. Skeletal and cardiac
78 muscles showed necrosis and regeneration of muscle fibers associated to progressive
79 replacement by fibrotic and adipose tissue. Weak muscle strength and muscle lesions therefor
80 closely mimic those observed in DMD patients. *Dmd*^{mdx} rats represent a useful small animal
81 model of pre-clinical research for DMD ⁴.

82 To date, there is no cure for muscular dystrophies, and despite that gene and cell therapies
83 will likely bring in the future cure of the disease there is still need for therapies for associated
84 pathology such as immune responses and inflammation. Immune responses are involved in
85 the pathophysiology of disease in both DMD patients and *mdx* mice [for a review see ⁵].
86 Standard therapy of DMD is based on treatment with corticosteroids (CS), which have been
87 shown to act at least in part through anti-inflammatory actions and inhibition of CD8⁺ T cells
88 that improve muscle strength in a fraction of patients ⁵⁻⁷. Apart from its moderate efficacy, CS
89 treatment is limited by serious systemic side effects, such as short stature, obesity,
90 psychological symptoms, osteoporosis, diabetes and hypertension ⁶. Furthermore, CS through

91 their broad and nonspecific anti-inflammatory effects inhibit inflammatory mechanisms that
92 promote muscle repair⁵.

93 T effector cells against DMD have been described in patients before and after gene therapy⁸⁻
94 ¹⁰. CD4⁺ T regulatory cells (Tregs) limit the severity of the disease in *mdx* mice not only
95 through inhibition of immune responses but also by their tissue repair activity^{5, 11, 12}.

96 Thus, inhibition of immune responses and promotion of immune tolerance are potentially
97 important adjuvants to the therapeutic arsenal to treat DMD patients but these
98 immunointerventions should at the same time preserve immune responses that promote
99 muscle regeneration as well as protection against pathogens and cancer cells. Knowledge of
100 immune responses in DMD patients and animal models are thus important for targeted
101 immunointerventions associated to other treatments such as gene or cell therapy. Furthermore,
102 immune responses can also be an obstacle to gene and cell therapy since in both situations
103 newly produced dystrophin could be recognized as immunogenic and cells expressing it
104 destroyed¹⁰. Thus, analyses of immune cells and immunotherapies in *Dmd^{mdx}* rats could give
105 potentially important results for development of new treatments for DMD patients.

106 We have described that CD4⁺ and CD8⁺ Tregs in rats and humans are comprised within
107 CD45RC^{low/-} cells^{13, 14}. We have recently shown that treatment with anti-CD45RC
108 monoclonal antibody (MAb) in a rat model of allograft rejection and in mouse immune
109 humanized models of graft versus host disease (GVHD) could induce permanent allograft
110 acceptance and inhibition of GVHD¹⁴. Anti-CD45RC treatment depleted only T cells that
111 were CD45RC^{high}, i.e. naïve T cells, precursors of Th1 cells and effector memory T cells
112 including TEMRA cells, whereas CD8⁺ or CD4⁺ Tregs, both in rats and humans, are
113 CD45RC^{low/-} and thus were spared. Among CD45RC^{low/-} cells, CD8⁺ and CD4⁺ Tregs specific
114 for donor alloantigens protect from graft rejection. Importantly, immune responses against
115 third party donors and exogenous antigens were preserved, thus anti-CD45RC antibody

116 treatment does not result in broad immunosuppression but rather specific elimination of T
117 cells with effector functions and preserved Tregs followed by their activation and expansion
118 ¹⁴.

119 We thus reasoned that treatment of *Dmd*^{mdx} rats with anti-CD45RC MAbs could eliminate
120 CD45RC^{high} effector T cells and enrich CD45RC^{low/-} Tregs. The later could then act at the
121 same time as inhibitors of immune responses and favoring muscle repair and homeostasis. To
122 the best of our knowledge, treatment with antibodies directed against other cell antigens that
123 favor immune tolerance in transplantation, GVHD or autoimmune diseases, such as anti-CD3,
124 -CD28, -CD127 or -CD137, have not been reported in none of the other animal models of
125 DMD.

126 We first analyzed immune parameters in *Dmd*^{mdx} rats and we secondly treated *Dmd*^{mdx} rats
127 with the same anti-CD45RC MAb previously used to induce allograft tolerance in comparison
128 to the standard of care (i.e. prednisolone). We observed that the skeletal and cardiac muscle of
129 *Dmd*^{mdx} rats showed a leukocyte infiltrate predominantly formed by macrophages and to a
130 lesser extent by T cells. M2 type macrophages increased with time. Treatment with an anti-
131 CD45RC depleting MAb resulted in increased muscle strength associated to a decrease in T
132 cells but not of macrophages. Prednisolone treatment also increased muscle strength and
133 decreased CD45RC^{high} cells but decreased growth of *Dmd*^{mdx} rats whereas anti-CD45RC did
134 not. CD45RC⁺ cells are also present in the blood and muscles of DMD patients.

135 Overall, immune responses and inflammation are present in the *Dmd*^{mdx} rat muscles and anti-
136 CD45RC MAb treatment resulted in amelioration of skeletal muscle strength. This is the first
137 report showing that a treatment with a monoclonal antibody targeting specific T cell
138 populations results in amelioration of clinical parameters in a pre-clinical model of DMD.

139

140 **Results.**

141 **Increased mononuclear leukocyte infiltration in skeletal muscles of *Dmd^{mdx}* rats.**

142 Mononuclear leukocytes in the muscle and spleen of *Dmd^{mdx}* rats were analyzed by flow
143 cytometry using the pan-leukocyte marker CD45 (**Fig 1**). The number of total mononuclear
144 leukocytes in the muscle of littermate wild type (WT) and *Dmd^{mdx}* rats were comparable at 2
145 weeks of age, but at 4 weeks *Dmd^{mdx}* rats showed a sharp increase that was maintained until
146 week 8 and then decreased at weeks 12 and 14 to values that were still significantly higher
147 than those observed in littermate WT rats (**Fig. 1A-B**). Granulocytes were rarely observed at
148 early time points in biopsies stained with Hemalun-Eosin-Saffron (data not shown). Total
149 leukocyte numbers in the spleen were comparable between WT and *Dmd^{mdx}* rats at all-time
150 points analyzed (**Fig. 1A**). Thus, limb muscles of *Dmd^{mdx}* rats showed an anatomical specific
151 leukocyte infiltrate that indicates the presence of a localized immune/inflammatory response.

152

153 **Presence of macrophages and T cells in skeletal muscle of *Dmd^{mdx}* rats as analyzed by**
154 **cytofluorimetry.**

155 We used flow cytometry analysis to obtain frequencies and absolute numbers, of different
156 mononuclear leukocyte populations. The analysis of viable CD45⁺ mononuclear leukocyte
157 subpopulations showed that ~90% of muscle infiltrating cells in *Dmd^{mdx}* rats were CD68⁺ (vs.
158 ~60% in WT rats), increasing sharply at 4 weeks, maximal at 8 weeks and decreased but were
159 still higher than WT at 12 and 16 weeks of age and were of higher granularity as shown by
160 their SSC profile (**Fig. 2A**). In contrast, numbers of spleen CD68⁺ macrophages increased
161 steadily with age and were comparable between *Dmd^{mdx}* and WT rats (**Fig. 2A**). Identical
162 results were obtained with the macrophage marker SIRP α (**Supplementary figure 1**).

163 Analysis of the M2 marker CD163 also showed a similar curve with an increase at 4 weeks,
164 maintained at 8 weeks and a decrease at 12 and 16 weeks of age with an increase in CD68
165 expression levels in some animals (**Fig. 2B**). In contrast, the number of CD163⁺ macrophages
166 in spleen increased with age and were comparable between *Dmd^{mdx}* rats and WT rats (**Fig.**
167 **2B**). The ratio of M2:M1 macrophages in muscles of *Dmd^{mdx}* rats was comparable at 4 weeks,
168 increased non significantly at 8 weeks and was significantly higher at 12 and 16 weeks of age,
169 whereas it was constant in muscles of WT rats (**Fig. 2C**). In the spleen, ratio of M2:M1
170 macrophages increased with time but was always lower than in muscles and comparable for
171 both *Dmd^{mdx}* and WT rats except at 16 weeks of age in which *Dmd^{mdx}* showed a modest but
172 significant increase vs. WT rats (**Fig. 2C**).

173 Analysis of T cells in muscles showed that total TCR⁺αβ cells (**Fig. 3A-B**) as well as CD4⁺
174 (**Fig.3 C-D**) and CD8⁺ T cells (**Fig. 3E-F**) in *Dmd^{mdx}* rats increased sharply at 4 weeks and
175 then decreased at later time points with significantly higher levels at 4 and 12 weeks vs. WT
176 animals. In contrast, in the spleen, total TCR⁺ cells, CD4⁺ T cells and CD8⁺ T cells for both
177 *Dmd^{mdx}* and WT rats increased steadily to comparable numbers at 8 weeks and remained
178 stable (**Fig. 3A-F**).

179 The muscles of *Dmd^{mdx}* vs. WT rats showed significantly increased levels of Foxp3⁺ CD4⁺
180 Tregs at 4 and 12 weeks (**Fig. 3G-H**). As we previously described^{15, 16}, CD8⁺ Tregs were
181 defined as CD8⁺CD45RC^{low} T cells and were significantly increased at 4 and 12 weeks (**Fig.**
182 **3I-J**). In contrast, in spleen, total Foxp3⁺CD4⁺ Tregs and CD8⁺CD45RC^{low} Tregs of both
183 *Dmd^{mdx}* and WT rats increased comparably at 8 weeks and remained stable (**Fig. 3H and J**).

184 B cells (CD45RA⁺ and CD45R⁺) and NK cells (CD161^{high}) represented respectively always
185 <2% and 3% of total muscle leukocytes in both *Dmd^{mdx}* and WT rats and were comparable in
186 spleens of both *Dmd^{mdx}* and WT rats (**data not shown**).

187 Thus, the majority of leukocytes in muscles of *Dmd^{mdx}* rats were macrophages that reached
188 maximal levels between 4 and 8 weeks of age and the ratio of M2:M1 increased at 12 and 16
189 weeks of age. T cells, including CD8⁺ and CD4⁺ Tregs, showed a similar evolution with
190 similar proportions of both CD4⁺ and CD8⁺ cells.

191

192 **Detection of macrophages in cardiac and skeletal muscle of *Dmd^{mdx}* rats as analyzed by**
193 **immunohistology.**

194 We used tissue immunofluorescence to analyze leukocyte populations in cardiac muscle since
195 flow cytometry analysis required numbers of cells higher than we could routinely obtain from
196 hearts from WT origin and to confirm the presence in skeletal muscle of leukocytes defined
197 by flow cytometry. Skeletal and cardiac muscle biopsies at 8 and 12 weeks of age showed the
198 presence of CD68⁺ and CD163⁺ macrophages but few CD3⁺ cells in connective tissue of both
199 skeletal and cardiac muscles of *Dmd^{mdx}* rats. In comparison, only a few CD68⁺ macrophages
200 were observed sporadically in WT rats (**Fig. 4**). CD163⁺ macrophages were notably numerous
201 in foci of mononuclear cell infiltration in the cardiac muscle. Thus, immunohistology of
202 skeletal muscles from *Dmd^{mdx}* rats confirmed results obtained by flow cytometry and revealed
203 very similar pattern in cardiac muscle. As previously described³, increased fibrosis (**Fig. 4**),
204 fiber necrosis and regeneration (**data not shown**) are present in skeletal and cardiac muscle of
205 *Dmd^{mdx}* as soon as 4 weeks and more severely at 8 weeks of age. Along with these lesions,
206 total creatinine kinase (CK) levels in serum, released from damaged muscle fibers, were
207 comparable at week 2 between *Dmd^{mdx}* and WT rats and then increased significantly in
208 *Dmd^{mdx}* rats to reach peak levels between weeks 4 and 8 and decreased at 12 weeks, returning
209 to normal levels at week 16 (**Supplementary figure 2**).

210 These results indicate that infiltration of muscle by leukocytes was associated to damaged
211 muscle fibers and elevated CK serum levels.

212

213 **Inflammatory and growth factors in leukocytes infiltrating muscle and serum of *Dmd^{mdx}***
214 **rats.**

215 We used quantitative RT-PCR to analyze mRNA levels of several molecules involved in the
216 initiation or suppression of immune responses and inflammation, as well as some muscle
217 trophic factors in isolated mononuclear leukocytes from muscles of *Dmd^{mdx}* and WT at 8 and
218 12 weeks of age (**Fig. 5A**). TNF α expression was particularly and strongly increased in
219 mononuclear cells from muscles of *Dmd^{mdx}* vs. WT at 8 weeks. Similarly, heme oxygenase-1
220 (HO-1), IFN γ , TGF β , IL-10 as well as the muscle trophic factor amphiregulin¹¹ were
221 significantly increased at 8 and/or 12 weeks (**Fig. 5A**). Arginase and IL-34 were decreased in
222 mononuclear cells from muscle of *Dmd^{mdx}* rats vs. WT rats, at weeks 8 and 12 respectively
223 (**Fig. 5A**). IL-6 and iNOs were not statistically different in *Dmd^{mdx}* vs. WT rats but the former
224 showed higher numerical levels in *Dmd^{mdx}* rats (**Fig. 5A**). Relaxin3 and indoleamine 2,3-
225 dioxygenase (IDO) were detectable at very low levels without differences among the different
226 groups of animals (**data not shown**).

227 To further evaluate cytokines in *Dmd^{mdx}* rats, we analyzed by using a multiplex assay the
228 presence of cytokines in the sera of animals at different time points. IL-1 β and IL-10 were
229 detectable in serum in both *Dmd^{mdx}* and WT rats without significant differences between them
230 at 8, 12 and 16 weeks and as compared to 2 weeks IL-10 was significantly elevated only at 12
231 weeks (**Fig. 5B**). TNF α and IL-6 levels were undetectable (**data not shown**).

232 Overall, several mediators of inflammation were increased in muscle or serum, such as TNF α
233 and IL-1 β , respectively, and several anti-inflammatory molecules, such as HO-1, TGF β ,
234 amphiregulin and IL-10 were increased in muscle, as well as the later also in serum.

235

236 **Treatment with anti-CD45RC MAb depleted CD45RC^{high} T cells and improved skeletal**
237 **muscle strength.**

238 Anti-CD45RC MAb treatment induces organ transplantation tolerance and inhibits GVHD at
239 least partially mediated by depletion of T CD8⁺CD45RC^{high} and CD4⁺CD45RC^{high} cells
240 involved in organ rejection and GVHD and in the organ transplantation model by increased
241 suppressor activity against donor antigens by CD8⁺CD45RC^{low/-} and CD4⁺CD45RC^{low/-} Tregs
242 ¹⁴. Since CD45RC expression levels can differ in different rat strains ¹⁷ and have not been
243 reported in muscle, we first analyzed the distribution of CD45RC^{high} and CD45RC^{low/-}
244 leukocytes within different leukocytes subsets in the muscle and spleen of *Dmd^{mdx}* and WT
245 Sprague-Dawley rats.

246 In muscles of *Dmd^{mdx}* rats, we observed that, within the CD8⁺ T cell population, absolute
247 numbers of CD45RC^{low/-} (**Fig. 3I-J**) and CD45RC^{high} cells (**Supplementary figure 3A**)
248 increased sharply and significantly in *Dmd^{mdx}* vs. WT at 4 weeks, remained elevated at 8
249 weeks and then decreased at 12 weeks to low levels observed in WT rats. Numbers in spleen
250 of CD45RC^{high} and CD45RC^{low/-} cells were comparable of both *Dmd^{mdx}* and WT rats
251 (**Supplementary figure 3A and Fig. 3I**).

252 For the TCR⁺CD4⁺ cell population absolute numbers of CD45RC^{low/-} cells increased
253 significantly at 4 and 12 weeks in the muscle of *Dmd^{mdx}* rats vs. WT and in the spleen
254 increased progressively without statistical differences (**Supplementary figure 3C-D**).

255 Absolute numbers of CD45RC^{high} cells in muscle of *Dmd*^{mdx} rats increased but not
256 significantly vs. WT rats and in the spleen there were no differences between *Dmd*^{mdx} and WT
257 rats (**Supplementary figure 3E-D**).

258 For the non-T cells, which were mostly macrophages, CD45RC^{low/-} increased significantly at
259 4 weeks, remained elevated at 8 weeks and decreased at 12 weeks. (**Supplementary figure**
260 **3F-G**). TCR⁻ CD45RC^{high} increased non significantly at 4 and 8 weeks, decreased at weeks 12
261 and 16 and are statistically higher in *Dmd*^{mdx} compared to WT rats (**Supplementary figure**
262 **3H-G**). In the spleen, TCR⁻ cells showed similar proportion of CD45RC^{high} and CD45RC^{low/-}
263 cells in *Dmd*^{mdx} and WT animals (**Supplementary figure 3F-H**).

264 WT and *Dmd*^{mdx} rats were injected with the same anti-CD45RC MAb used in the
265 transplantation model described above¹⁴ from week 2, since at this time point the leukocyte
266 infiltration into the muscle has not yet appeared, and every 3.5 days and up to week 12 when
267 grip force and mononuclear cells from muscle and spleen were analyzed.

268 At 12 weeks of age, treatment with anti-CD45RC MAb significantly depleted
269 CD8⁺CD45RC^{high} T cells in both muscle and spleen of *Dmd*^{mdx} and in spleen of WT rats
270 whereas CD8⁺CD45RC^{low/-} T cells were unchanged in both muscle and spleen (**Fig. 6A**).

271 Numbers of CD4⁺CD45RC^{high} T cells in the spleen were decreased but it did not reach
272 statistical significance (**Fig. 6B**). CD4⁺CD45RC^{low/-} (**Fig. 6B**) and FoxP3⁺ CD4⁺ T cells (data
273 not shown) were maintained in both muscles and spleen. As in the transplantation models,
274 other leukocytes that are CD45RC^{high} and TCR⁻, such as macrophages and B cells were not
275 depleted by anti-CD45RC treatment (**Fig. 6C**).

276 At week 12 the animals were analyzed using a grip test. As previously reported³, *Dmd*^{mdx} rats
277 had a 30% reduction in forelimb strength compared to WT littermates (**Fig. 6D**). The
278 treatment with anti-CD45RC MAb significantly improved muscle strength in *Dmd*^{mdx} treated
279 rats vs. *Dmd*^{mdx} control animals (**Fig. 6D**). Furthermore, values for *Dmd*^{mdx} rats treated with

280 anti-CD45RC MAb were indistinguishable of those of littermate WT controls (**Fig. 6D**),
281 despite that they showed a significantly lower strength vs. WT animals treated with anti-
282 CD45RC, but this was due to a slight non-significantly increase in muscle strength of WT
283 animals treated with anti-CD45RC vs. WT isotype control-treated animals (**Fig. 6D**). The
284 weight gain curves of *Dmd*^{mdx} animals were lower as compared to WT animals and treatment
285 with anti-CD45RC neither modified this curve (**Fig. 6E**), nor the general aspect of the skeletal
286 muscle fibrosis (**Supplementary figure 4A-B**) and CK levels in serum (**Supplementary**
287 **figure 4C**).

288 Thus, anti-CD45RC treatment resulted in increased muscle strength in *Dmd*^{mdx} rats and it was
289 associated to depletion of T CD8⁺CD45RC^{high} cells.

290

291 **Treatment with prednisolone improved skeletal muscle strength but had secondary**
292 **effects.**

293 Since CS are standard treatment in DMD patients ⁶, we analyzed the clinical effect of
294 prednisolone on muscle strength of *Dmd*^{mdx} rats as well as in immune cells in skeletal muscle
295 and spleen of *Dmd*^{mdx} rats.

296 Prednisolone-treated rats also showed at 12 weeks of age a significant decrease of
297 CD8⁺CD45RC^{high} T cells in both muscle and spleen of *Dmd*^{mdx} rats and in spleen of WT rats
298 (**Fig. 7A**). CD8⁺CD45RC^{low/-} T cells were maintained (**Fig. 7A**). CD4⁺CD45RC^{high} T cells
299 were significantly decreased in spleen but not in muscle (**Fig. 7B**). CD4⁺CD45RC^{low} T cells
300 were also decreased in the spleen but not in the muscle (**Fig. 7B**). Other leukocytes that are
301 CD45RC^{high} and TCR⁻, such as macrophages and B cells were not depleted by prednisolone
302 treatment (**Fig. 7C**).

303 Simultaneously, *Dmd*^{mdx} rats treated with prednisolone showed significantly increased muscle
304 strength at 12 weeks to levels identical to those of WT or anti-CD45RC-treated rats (**Fig. 7D**).
305 Prednisolone-treated *Dmd*^{mdx} rats showed marked secondary effects, as shown by a severe
306 (25%) and significant reduction in growth as compared to WT rats but also to NaCl-treated
307 *Dmd*^{mdx} rats (**Fig. 7E**). Prednisolone had no effect on the growth of WT animals (**Fig. 7E**).
308 Muscle tissue fibrosis (**Supplementary figure 4A-B**) and CK levels in serum
309 (**Supplementary figure 4C**) were not modified by prednisolone treatment.

310 Thus, as compared to anti-CD45RC treatment, prednisolone also increased muscle strength
311 but showed a larger decrease in cell populations including not only CD8⁺CD45RC^{high} cells in
312 muscle and spleen, but also CD4⁺CD45RC^{high} and CD4⁺CD45RC^{low} cells in spleen and had a
313 strong negative effect on the growth of *Dmd*^{mdx} animals.

314

315 **Presence of T CD45RC^{high} cells in skeletal muscles and blood of DMD patients.**

316 To further explore the potential of CD45RC as an immunotherapeutic target, we evaluated the
317 presence of CD45RC^{high} cells in peripheral blood in DMD patients. Cytofluorimetry analysis
318 showed the presence of CD45RC^{high} and CD45RC^{low/-} among both CD4⁺ or CD8⁺ T cell
319 compartments in blood of DMD patients in proportions comparable to those of age-matched
320 young individuals hospitalized for pathologies not involving the immune system or other
321 neuromuscular diseases (**Supplementary figure 5**). As for young controls, B and NK cells
322 from DMD patients were all CD45RC^{high} whereas monocytes and PMN were all CD45RC⁻
323 (**Supplementary figure 6**).

324 Furthermore, the presence of CD45RC brightly positive cells was confirmed in muscle
325 biopsies from DMD patients and not of normal individuals, as it was the case in muscles of
326 *Dmd*^{mdx} vs. WT animals (**Fig. 8**).

327

328

329 **Discussion.**

330 DMD patients and *mdx* mice show muscle infiltration by different types of leukocytes and
331 production of a variety of mediators that have been shown to play facilitating or protecting
332 roles in the evolution of the disease⁵. Not only inflammation and innate immune responses
333 are present, but also adaptive immune responses including anti-dystrophin T cells and Treg
334 cells are involved in DMD patients^{8,10} and *mdx* mice^{5,11,12}. CS are one of the only standard
335 treatments that DMD patients receive and that prolong ambulation by about 2 years.
336 Nevertheless, increase muscular strength responses are variable, incomplete and always
337 associated to serious side effects^{6,7}. Despite that the precise mechanisms of action of CS in
338 DMD patients are ill defined, anti-inflammatory effects are likely important^{6,7}. Thus, there
339 are unmet clinical needs to treat the inflammatory and immune effects caused by dystrophin
340 deficiency while awaiting for curative gene or stem cell therapies. It is even very likely that
341 these immunotherapies will be associated to gene and cell therapy to inhibit immune
342 responses against the vectors, transgene products or antigenic cellular products.

343 The *mdx* mouse is a very useful model but fails to reproduce key symptoms of DMD patients
344 such as muscular weakness². Thus, although several immunotherapies were successful in *mdx*
345 mice, such as intravenous immunoglobulin¹⁸, anti-TNF α antibodies¹⁹, IL-6 blocking²⁰,
346 tranilast²¹, heme oxygenase-1 (HO-1) inducers²², IL-1 receptor antagonist²³ and IL-2
347 complexes to amplify CD4⁺ Tregs¹², their potential effect in DMD patients is uncertain.
348 *Dmd*^{*mdx*} rats reproduce skeletal and cardiac muscular weakness at early time points and
349 develop skeletal and cardiac muscle tissue lesions that resemble those observed in DMD
350 patients^{3,4}. In the present manuscript we describe that *Dmd*^{*mdx*} rats present mononuclear cells
351 infiltrating both skeletal and cardiac muscles that appeared early, between 2 and 4 weeks of
352 age, and that had greatly decreased by 16 weeks of age. The majority of these mononuclear
353 cells were CD68⁺ and SIRP α ⁺ macrophages and the proportion of M2 CD163⁺ increased with

354 time. Macrophages appear early in both *mdx* mice (2 weeks) and DMD patients (2-year-old)
355 ²⁴. M2 macrophages have been shown to play protective and regenerative roles in early stage
356 disease in *mdx* mice ⁵. CD4⁺ and CD8⁺ T cells, including Tregs, were also increased in
357 muscles of *Dmd*^{*mdx*} rats compared to controls. The lesions of muscular fibers, as analyzed by
358 CK levels in serum, followed the kinetics of leukocyte infiltration, with normal levels at 2
359 weeks of age and a peak between 4 and 8 weeks of age for a later decrease, possibly reflecting
360 a more pronounced immune attack at early rather than late time points.

361 Cytokines were produced at increased levels by mononuclear cells from *Dmd*^{*mdx*} rats
362 compared to controls at 8 and/or 12 weeks of age, including IL-1 β and TNF α . These
363 cytokines are increased in DMD patients and *mdx* mice, have been described as potential
364 immunotherapy targets ²⁵, since anti-TNF α treatment reduces early muscle damage in *mdx*
365 mice ¹⁹ could be targeted in the future in *Dmd*^{*mdx*} rats. Several anti-inflammatory molecules,
366 such as HO-1, IL-10 and TGF β , as well as the muscle trophic factor amphiregulin ¹¹, were
367 also produced, likely as a response to inflammation and ongoing immune responses, as
368 previously described in *mdx* mice and DMD patients ⁵. Inhibition of TGF β has been shown to
369 play a dual role since early neutralization in *mdx* mice decreases fibrosis but increases T cell
370 infiltration and inflammation²⁶.

371 We have recently shown that treatment with an anti-CD45RC MAb in a rat model of heart
372 allograft rejection could induce permanent allograft acceptance ¹⁴. Furthermore, anti-CD45RC
373 MAb treatment prevented GVHD in immune humanized NSG (NOD *Scid* Gamma) mice ¹⁴.
374 Anti-CD45RC treatment depleted T cells that were CD45RC^{high}, comprising naïve T cells,
375 precursors of Th1 cells and T effector memory cells including TEMRA cells, whereas CD8⁺
376 and CD4⁺ Tregs both in rats and humans are CD45RC^{low/-} ^{13, 27} and thus were spared. These
377 CD45RC^{low/-} CD8⁺ and CD4⁺ Tregs that were specific of donor alloantigens could impose

378 allograft tolerance in newly grafted irradiated recipients following adoptive cell transfer.
379 Importantly, immune responses against third party donors and exogenous antigens were
380 preserved during treatment with anti-CD45RC, thus depletion of CD45RC^{high} cells does not
381 inhibit all immune responses.

382 We thus reasoned that treatment of *Dmd*^{mdx} rats with anti-CD45RC MAbs could eliminate
383 CD45RC^{high} T effector cells and their precursors and enrich CD45RC^{low/-} Tregs that could then
384 act at the same time, not only as inhibitors of immune responses by CD45RC^{high}, but also
385 favor tissue repair and homeostasis by CD45RC^{low/-}, such as it has been described for CD4⁺
386 Tregs both in muscle ¹¹ and adipose tissue ²⁸. In the present manuscript we show that anti-
387 CD45RC treatment improved muscle strength to the levels of WT animals and that this was
388 associated to a depletion of CD8⁺ CD45RC^{high} T cells at 12 weeks. CD4⁺ CD45RC^{high} T
389 effector cells were decreased but not significantly at this time of treatment. Although
390 CD45RC^{low/-} CD8⁺ or CD4⁺ Tregs were not numerically increased in *Dmd*^{mdx} rats this was
391 also the case in rats that were tolerant to transplanted organs after anti-CD45RC MAb
392 treatment ¹⁴. Whether their function is increased and play a role in the amelioration of muscle
393 strength observed in these animals remains to be analyzed in future studies.

394 As for the anti-CD45RC treatment, corticosteroids resulted in a similar increase in muscular
395 strength that was associated surprisingly to a specific decrease in CD8⁺ CD45RC^{high} T cells in
396 muscle but also to a more widespread decrease of CD4⁺CD45RC^{high} and CD4⁺CD45RC^{low/-}
397 cells in spleen. This effect has also been observed in DMD patients treated with steroids ⁵.
398 DMD patients treated with corticosteroids showed decreased T cells against dystrophin ¹⁰.

399 The secondary effects of steroids were observed in *Dmd*^{mdx} rats, whereas anti-CD45RC
400 treated animals did not show obvious clinical abnormalities and no weight loss. Since patients
401 suffer from several important side effects of steroids, anti-CD45RC treatment could result in

402 similar muscle improvement than corticosteroids but without side effects. A potential side
403 effect of anti-CD45RC treatment could be generalized immunosuppression but we have
404 already demonstrated that rats treated with anti-CD45RC treatment could mount normal
405 primary immune responses to new antigens as well as memory immune responses after
406 secondary immunization ¹⁴.

407 MAbs against CD45RA ²⁵, CD45RO/B ²⁹ and CD45RB ³⁰ have been used to treat organ
408 rejection and/or GVHD but none has been used as isolated treatment, neither in animal
409 models of DMD, nor on animal models of muscle lesions. Even if anti-CD45RA or
410 antiCD45RO/B could be used in the future, since between 50 to 90% of both of the CD8⁺ and
411 CD4⁺ Tregs are CD45RA^{high} and CD45RB^{high} ¹⁴, the outcome of treatment with anti-CD45RC
412 is clearly targeting different cell populations and thus distinct and likely more favorable since
413 it preserves Tregs. Although in mdx mice depletion of total CD4⁺ or CD8⁺ cells ameliorates
414 histopathology ³¹, none of other tolerizing treatments based on MAbs and used in organ
415 transplantation, GVHD or autoimmunity, such as anti-CD3, anti-CD127, anti-CD28, have
416 been previously used in models of DMD and thus the results in the present manuscript could
417 stimulate the use of these other reagents.

418

419 **Materials and methods.**

420 **Animal experiments and ethical aspects.**

421 *Dmd*^{mdx} rats have been previously described³. *Dmd*^{mdx} and wild-type littermate were raised in
422 SPF conditions. All the animal care and procedures performed in this study were approved by
423 the Animal Experimentation Ethics Committee of the Pays de la Loire region, France, in
424 accordance with the guidelines from the French National Research Council for the Care and
425 Use of Laboratory Animals (Permit Numbers: CEEA-PdL-10792 and CEEA-PdL-8986). All
426 efforts were made to minimize suffering. The rats were housed in a controlled environment
427 (temperature 21±1°C, 12-h light/dark cycle). Blood samples from 2 DMD patients were
428 obtained as part of their standard care management in the hospital and after obtaining
429 informed consent from both patients and their parents. Control blood samples were collected
430 from children who had been admitted in Nantes University Hospital without immune
431 deficiency. The biocollection used for this analysis is the "pediatrics" collection (Ref: MESR
432 DC-2011-1399) which is a prospective monocentric collection managed by the University
433 Hospital of Nantes and approved by the local ethics committee. None of the legal
434 representatives of the children objected to let them take part in this biocollection. Tissue
435 samples were obtained from the *Paravertebralis* muscle of four 12 year-old patients (two
436 DMD patients and two patients free of known muscular disease). Patients were operated at the
437 Department of Pediatric Surgery of the Centre Hospitalier Universitaire (CHU) de Nantes
438 (France). They gave written informed consent. All protocols were approved by the Clinical
439 Research Department of the CHU (Nantes, France), according to the rules of the French
440 Regulatory Health Authorities (Permit numbers: MESR/DC-2010-1199). Biological sample
441 bank was constituted in compliance with the national guidelines regarding the use of human
442 tissue for research (Permit numbers: CPP/29/10).

443

444 **Preparation of muscle and spleen single-cell suspensions.**

445 Muscles of both hindlimbs from WT or *Dmd^{mdx}* rats were excised without adipose tissue,
446 rinsed with PBS and weighed. Muscles were minced, placed in gentleMACS C tubes
447 (Miltenyi Biotec) with collagenase D (4ml/g of muscle), dissociated using the gentleMACS™
448 dissociator (gentleMACS program “m_muscle_01”) and incubated for two runs of 30 min at
449 37°C under continuous rotation. After the initial run, undigested muscle was filtrated on a
450 mesh strainer and the resulting cell suspension was centrifuged and resuspended in PBS FCS
451 2% 1 mM EDTA. The remaining undigested muscle was further incubated in fresh
452 collagenase for a new run of 30 min. The debris-free cell suspensions were centrifuged,
453 resuspended in PBS FCS 2% 1 mM EDTA, and pooled with cells from the first digestion.
454 Pooled cells were then applied to 15 ml Histopaque 1077 density gradient (Eurobio) and
455 centrifuged at 1000 x g for 30min. The cells at the interface were collected, washed,
456 resuspended in PBS FCS 2% 1mM EDTA and counted.

457 Spleen was harvested, perfused with collagenase D, minced and incubated for 15 min at 37°C.
458 Spleen fragments were then scraped in the presence of PBS FCS 2% 1mM EDTA and
459 mononuclear cells were recovered using a density gradient (Histopaque 1077, Eurobio). The
460 cells at the interface were collected, washed, resuspended in PBS FCS 2% 1mM EDTA and
461 counted.

462

463 **Staining of rat cells for flow cytometry analysis.**

464 Cytofluorimetry analysis was performed as previously described in detail ³². Briefly, single-
465 cell suspensions from muscle or spleen were stained with MAbs against the following
466 antigens : CD45 as a pan leukocyte (clone OX-1), TCRαβ (clone R7/3), CD45RA in B cells
467 (clone OX33), CD45R/B220 in B cells (clone His24), anti-granulocytes (RP-1 and His48),
468 CD4 (clone w3/25), CD45RC (clone OX22 or clone OX32), CD25 (clone OX39), CD8 (clone

469 OX8), CD172a/SIRP α (clone OX41), CD161 in NK and myeloid cells (clone 3.2.3), CD163
470 in macrophages (clone ED2), CD68 for macrophages (clone ED1) and with viability dye
471 eFluor506 or eFluor450 from eBiosciences to assess cell viability. Analysis was performed on
472 a BD FACS Verse with FACSuite Software version 1.0.6. Post-acquisition analysis was
473 performed with FlowJo software.

474

475 **Serum creatinine phosphokinase and cytokine levels.**

476 Blood was collected under anesthesia, serum was isolated and immediately frozen at -20°C.
477 Total creatinine phosphokinase (CK) activity was determined in the biochemistry department
478 of Nantes University Hospital.

479 Levels of IL-1 β , IL-6, IL-10 and TNF α in the serum of *Dmd*^{mdx} or WT littermate rats, were
480 measured by multiplex assay (Luminex technology) (R&D systems) following to the
481 manufacturer's instructions.

482

483 **Quantitative RT-PCR.**

484 Quantification of mRNA levels was performed as previously described in detail³³. Briefly,
485 total RNA extraction has been performed on mononuclear cells from skeletal muscles using
486 RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Then quantification
487 and quality analysis were done on Caliper LabChip GX II (PerkinElmer). RNA with a quality
488 score between 7 and 10 were retro-transcribed using oligo-dT and M-MLV reverse
489 transcriptase (Life Technologies). Fast SybrGreen Master Mix 2x was used to performed
490 qPCR on ViiA 7 (Applied Biosystems) on cDNA in duplicate for each target according to the
491 manufacturer's instructions. qPCR reaction conditions were 20 seconds at 95°C followed by
492 40 cycles of 1 second at 95°C, 20 seconds at 60°C and 20 seconds at target melting

493 temperature minus 3°C, ended by a melting curve stage. Calculations were made by DDCt
494 method. The primers used in this study are listed in **table 1**.

495

496 **Immunohistological analysis and fibrosis quantification.**

497 Immunohistochemistry was performed as previously described in detail ³. Briefly, tissue
498 samples of *Biceps femoris* and cardiac ventricular muscles were harvested at 8 and 12 weeks
499 of age and frozen and 8-µm-thick sectioned for immunofluorescence labelling. Sections were
500 preliminary fixed with acetone for CD3 labelling and with acetone (30%) in methanol for
501 CD68, CD163 and CD45RC labelling (10 min, room temperature) and incubated with 0.2%
502 triton in PBS (10 min, room temperature). Sections were then blocked with 10% goat serum
503 in PBS and incubated with the primary antibodies. Rabbit polyclonal antibody for CD3
504 (DakoCytomation, Glostrup, Denmark), mouse monoclonal antibodies for rat CD68, CD163
505 and CD45RC were used respectively at 1:50; 1:200 and 1:200 (overnight, 4°C). After
506 washing, goat anti-rabbit and goat anti-mouse antibodies coupled with Alexa 488
507 (InVitroGen, Carlsbad, CA) were respectively used to reveal CD3 and CD68 primary
508 antibody (1 h, room temperature). Section were incubated with wheat germ agglutinin Alexa
509 Fluor 555 conjugate for connective tissue labelling (Molecular Probes, Eugene, OR)
510 diluted 1:700 in PBS (overnight, 4°C) and nuclei were then labelled with Draq5 (BioStatus
511 Ltd, Shepshed, UK) diluted at 1:1000 (10min, room temperature). Immunofluorescence
512 labeling was analyzed with a laser scanning confocal microscope (Zeiss, LSM880, Jena,
513 Germany).

514 For human muscle, biopsies were obtained from DMD patients undergoing surgery for spinal
515 deformities and from young individuals undergoing muscle biopsy for other diagnosis. Tissue
516 was frozen, sectioned and processed as described above for rat tissue using an anti-human
517 CD45RC MAb (BD Biosciences).

518

519 **Treatment with anti-CD45RC and prednisolone.**

520 WT and *Dmd*^{mdx} rats received intraperitoneal injections of the anti-rat CD45RC MAb (clone
521 OX22, mouse IgG1) or an isotype control MAb (clone 3G8, mouse IgG1) at 1.5 mg/kg, every
522 3.5 days from week 2 to week 12 of age as previously described ¹⁴. Prednisolone was
523 administered by daily intraperitoneal injections of 0.5 mg/kg, close to the dose of 1 mg/kg in
524 mdx mice ³⁴ and 0.75 mg/kg in DMD patients ³⁵ from week 2 to week 12 of age.

525

526 **Grip test.**

527 Grip test was performed as previously described in detail ³. Rats were placed with their
528 forepaws on a grid and were gently pulled backward until they released their grip, as
529 previously described. A grip meter (Bio-GT3, BIOSEB, France), attached to a force
530 transducer measured the peak force generated.

531

532 **Statistical analysis.**

533 Mann–Whitney t test was used to compare numbers of cells in muscle and spleen of WT vs
534 *Dmd*^{mdx}, CK and cytokine levels in sera.

535 Two-way ANOVA test was used to compare growth curves.

536

537

538

539 **Figure legends.**

540

541 **Figure 1. Number of leukocytes in skeletal muscle and spleen of *Dmd^{mdx}* rats.** Hind limb
542 muscles and spleen were harvested from littermate wild-type (WT) or *Dmd^{mdx}* (KO) rats at the
543 indicated time points of age. Muscles and spleens were digested with collagenase,
544 mononuclear cells were isolated using a density gradient and analyzed by cytofluorimetry. **A)**
545 Number of viable CD45⁺ cells per gram of muscle (left panel) or whole spleen (right panel) at
546 different time points. WT, n=4, 5, 7, 7, 9 at 2, 4, 8, 12 and 16 weeks, respectively; *Dmd^{mdx}*,
547 n=3, 6, 10, 11, 16, at 2, 4, 8, 12 and 16 weeks, respectively * p< 0.05, ** p< 0.01, and *** p<
548 0.001. **B)** Representative dot-plot analysis of viable SSC CD45⁺ mononuclear leukocytes
549 from muscle (left panel) or spleen (right panel) from animals at 12 weeks of age.

550

551 **Figure 2. Macrophages in skeletal muscle and spleen of *Dmd^{mdx}* rats.** Cytofluorimetry of
552 single-cell suspensions from hind limb muscles or spleen WT or *Dmd^{mdx}* (KO) at the indicated
553 time points of age. **A)** Total number of macrophages CD68⁺ cells per gram of muscle (upper
554 left panel) or of whole spleen (upper right panel). Representative dot plots of macrophages
555 high granularity using side scatter (SSC^{high}) CD68⁺ cells after gating on viable (negatively-
556 stained cells) CD45⁺ cells from muscle of WT or *Dmd^{mdx}* 12 weeks-old rats (lower panel). **B)**
557 Total number of viable CD68⁺CD163⁺ type 2 macrophages per gram of muscle (upper left
558 panel) or whole spleen (upper right panel). Representative dot plots of viable CD68⁺CD163⁺
559 cells from muscle of WT or *Dmd^{mdx}* 12 weeks-old rats (lower panels). **C)** Macrophages type 2
560 (CD68⁺CD163⁺) over type 1 macrophages (CD68⁺CD163⁻) ratios in muscle (left panel) or
561 spleen (right panel) of WT (black) or *Dmd^{mdx}* (grey) rats. n=3, 6, 6, 7 and 8 (at 2,4, 8, 12 and
562 16 weeks of age, respectively) for *Dmd^{mdx}* rats and n= 4, 6, 4, 3 and 4 (at 2,4, 8, 12 and 16

563 weeks of age, respectively) for WT rats. * $p < 0.05$, *** $p < 0.01$. Results were obtained from
564 several experiments performed using all groups of animals in each experiment.

565

566 **Figure 3. T cells in skeletal muscle and spleen of *Dmd^{mdx}* rats.** Hind limb muscles or spleen
567 from WT or *Dmd^{mdx}* (KO) at the indicated time points of age were harvested, collagenase
568 digested and analyzed by cytofluorimetry. **A)** Total numbers of viable CD45⁺TCR⁺ cells per
569 gram of muscle (left panel) and of total spleen (right panel). **B)** Representative dot plots of
570 viable CD45⁺TCR⁺ cells from muscle of WT or *Dmd^{mdx}* 12 weeks-old rats. **C)** Total number
571 of CD45⁺TCR⁺CD4⁺ cells per gram of muscle (left panel) and of total spleen (right panel). **D)**
572 Representative dot plots of WT or *Dmd^{mdx}* 12 weeks-old rat muscle single-cell suspension
573 showing gating on viable CD45⁺TCR⁺CD4⁺ cells. **E)** Total number of TCR⁺CD8⁺ cells per
574 gram of muscle (left panel) and of total spleen (right panel). **F)** Representative dot plots of
575 WT or *Dmd^{mdx}* 12 weeks-old rat muscle single-cell suspension showing gating on viable
576 CD45⁺TCR⁺CD8⁺ cells. **G)** Total number of TCR⁺CD4⁺CD25⁺Foxp3⁺ cells per gram of
577 muscle (left panel) and whole spleen (right panel). **H)** Representative dot plots of WT or
578 *Dmd^{mdx}* 12 weeks-old rat muscle single-cell suspension showing gating on viable
579 CD45⁺TCR⁺CD4⁺CD25⁺Foxp3⁺ cells. **I)** Total number of TCR⁺CD8⁺CD45RC^{low/-} cells per
580 gram of muscle (left panel) and whole spleen (right panel). **J)** Representative dot plots of WT
581 or *Dmd^{mdx}* 12 weeks-old rat muscle single-cell suspension showing gating on viable
582 CD45⁺TCR⁺CD8⁺CD45RC^{low/-} cells. n=3, 6, 10, 12 and 4 (at 2, 4, 8, 12 and 16 weeks of age,
583 respectively) for *Dmd^{mdx}* rats and n= 4, 5, 7, 7 and 4 (at 2, 4, 8, 12 and 16 weeks of age
584 respectively) for WT rats. Results were obtained from several experiments performed using
585 all groups of animals in each experiment.

586

587 **Figure 4. Immunohistochemical detection of leukocytes in skeletal and cardiac muscle of**
588 ***Dmd^{mdx}* rats.** Skeletal muscle (*Biceps femoris*) and cardiac muscle were harvested at 8 and 12
589 weeks of age from wild-type (WT) and *Dmd^{mdx}* (KO) rats. **A)** Tissue sections were stained
590 with Draq5 to label nuclei (blue), with wheat germ agglutinin for connective tissue (red) and
591 with MAbs for detection of cells expressing CD3, CD68 or CD163 (green). Scale bar
592 identical for all pictures: 100 μ m.

593

594 **Figure 5. Inflammation markers and growth factors in skeletal muscle of *Dmd^{mdx}* rats.**
595 **A)** Mononuclear cells from skeletal muscles were harvested at 8 and 12 weeks of age from
596 wild-type (WT) and *Dmd^{mdx}* (KO) rats. Total RNA was extracted and mRNA levels for the
597 indicated molecules were analyzed by quantitative RT-PCR. * $p < 0.05$. **B)** IL1 β (left panel)
598 and IL10 (right panel) levels in the sera of *Dmd^{mdx}* (n=11, 3, 10, 5 at 2, 8, 12 and 16 weeks of
599 age, respectively) or WT (n= 12, 2, 5, 6 at 2, 8, 12 and 16 weeks of age, respectively) rats. *
600 $p < 0.05$.

601

602 **Figure 6. Effect of treatment with anti-CD45RC on lymphoid cell populations, forelimb**
603 **muscle strength and animal growth.** Hind limb muscles or spleen from WT or *Dmd^{mdx}*
604 (KO) rats were harvested at 12 weeks of age, collagenase digested and analyzed by
605 cytofluorimetry. **A)** Total numbers of viable CD45⁺TCR⁺CD8⁺CD45RC^{high} cells (upper
606 panels) or viable CD45⁺TCR⁺CD8⁺CD45RC^{low/-} (lower panels) cells per gram of skeletal
607 muscle (left panels) and of total spleen (right panels). **B)** Total numbers of viable
608 CD45⁺TCR⁺CD4⁺CD45RC^{high} cells (upper panels) or viable CD45⁺TCR⁺CD4⁺CD45RC^{low/-}
609 (lower panels) cells per gram of skeletal muscle (left panels) and of total spleen (right panels).
610 **C)** . Total numbers of viable CD45⁺TCR⁻ CD45RC^{high} cells per gram of skeletal muscle (left
611 panels) and of total spleen (right panels). **D)** Muscle strength in *Dmd^{mdx}* rats after treatment

612 with an anti-CD45RC MAb. Wild-type (WT) or *Dmd^{mdx}* rats received intraperitoneal
613 injections of the anti-rat CD45RC MAb (clone OX22, 1,5 mg/kg, every 3.5 days) or isotype
614 control Mab (clone 3G8, 1,5 mg/kg, every 3.5 days) from week 2 to week 12 of age when
615 muscle strength was analyzed using a grip test. Each point represents a single animal analyzed
616 in two different experiments. * $p < 0.05$. Results were obtained from several experiments
617 performed using all groups of animals in each experiment. **E)** Weight curves for animal
618 growth were determined serially. **** $p < 0.001$ between *Dmd^{mdx}* and WT rats for both
619 treatments but no difference between *Dmd^{mdx}* rats treated with anti-CD45RC vs. isotype
620 control.

621

622 **Figure 7. Treatment with prednisolone on lymphoid cell populations and forelimb**
623 **muscle strength.** Wild-type (WT) or *Dmd^{mdx}* (KO) rats received from week 2 of age
624 intraperitoneal injections of prednisolone (0.5 mg/kg, 5 days per week) or NaCl up to week
625 12. **A)** Hind limb muscles or spleen from WT or *Dmd^{mdx}* were harvested, collagenase
626 digested and analyzed by cytofluorimetry. Total numbers of viable
627 CD45⁺TCR⁺CD8⁺CD45RC^{high} cells (upper panels) or viable CD45⁺TCR⁺CD8⁺CD45RC^{low/-}
628 (lower panels) cells per gram of muscle (left panels) and of total spleen (right panels). * $p <$
629 0.05. **B)** Total numbers of viable CD45⁺TCR⁺CD4⁺CD45RC^{high} cells (upper panels) or viable
630 CD45⁺TCR⁺CD4⁺CD45RC^{low/-} (lower panels) cells per gram of muscle (left panels) and of
631 total spleen (right panels). **C)** Total numbers of viable CD45⁺TCR⁻ CD45RC^{high} cells per
632 gram of skeletal muscle (left panels) and of total spleen (right panels). **D)** Muscle strength
633 was analyzed using a grip test. Each point represents a single animal analyzed in two different
634 experiments. * $p < 0.05$. Results were obtained from several experiments performed using all
635 groups of animals in each experiment. **E)** Weight curves for animal growth were determined

636 serially. **<0.01 and ****<0.0001 for *Dmd*^{mdx} and WT with NaCl and prednisolone but
637 importantly ***p<0.001 between *Dmd*^{mdx} rats NaCl vs. prednisolone.

638

639 **Figure 8. CD45RC⁺ cells in rat and human dystrophin-deficient skeletal muscles.**

640 Skeletal muscle samples from rat (*Biceps femoris*) and humans (*Paravertebralis*), either from
641 dystrophin deficient individuals (n=2) or without muscle pathology (n=2). Pictures are
642 representative images of frozen tissue sections probed with Draq5 to label nuclei and with
643 anti-rat or human anti-CD45RC MAbs (green).

644

645

646 **Supplementary figure 1. Number of SIRP α ⁺ macrophages in skeletal muscle and spleen**
647 **of *Dmd*^{mdx} rats.** Hind limb muscles and spleen were harvested from littermate wild-type
648 (WT) or *Dmd*^{mdx} (KO) rats at the indicated time points of age. Muscle and spleen were
649 digested with collagenase, mononuclear cells were isolated using a density gradient and
650 analyzed by cytofluorimetry. **A)** Number of viable CD45⁺TCR⁻CD45RA⁻SIRP α ⁺ cells per
651 gram of muscle (left panel) or whole spleen (right panel) at different time points. WT, n=4, 5,
652 7, 7, 9 at 2, 4, 8, 12 and 16 weeks, respectively; *Dmd*^{mdx}, n=3, 6, 10, 11, 16 at 2, 4, 8, 12 and
653 16 weeks, respectively. ** p< 0.01, and *** p< 0.001. **B)** Representative dot-plot analysis of
654 viable SSC CD45⁺TCR⁻CD45RA⁻SIRP α ⁺ cells mononuclear leukocytes from muscle (left
655 panels) or spleen (right panels) from animals at 12 weeks of age.

656

657 **Supplementary figure 2. CK in sera of *Dmd*^{mdx} rats.** CK levels were determined
658 simultaneously in all samples. WT (n=8, 6, 6, 9, 5 at 2, 4, 8, 12 and 16 weeks, respectively),
659 *Dmd*^{mdx} (n=5, 4, 8, 8, 6 at 2, 4, 8, 12 and 16 weeks, respectively). * p<0.05.

660

661 **Supplementary figure 3. Expression profiles of CD45RC in different mononuclear cell**
662 **populations.** Hind limb muscles and spleen were harvested from littermate wild-type (WT) or
663 *Dmd*^{mdx} (KO) rats at the indicated time points of age. Muscle and spleen were digested with
664 collagenase, mononuclear cells were isolated using a density gradient and analyzed by
665 cytofluorimetry. **A)** Absolute numbers of CD45⁺CD45RA⁻TCR⁺CD8⁺CD45RC^{high} cells in
666 muscle or spleen of WT or *Dmd*^{mdx} rats during time. **B)** Representative dot-plot analysis of
667 viable CD45⁺CD45RA⁻TCR⁺CD8⁺CD45RC^{high} or ^{low/-} mononuclear leukocytes from muscle
668 (left panels) or spleen (right panels) of WT or *Dmd*^{mdx} rats from animals at 12 weeks of age.
669 **C)** Absolute numbers of CD45⁺CD45RA⁻TCR⁺CD4⁺CD45RC^{low/-} cells in muscle of spleen of
670 WT or *Dmd*^{mdx} rats during time. **D)** Representative dot-plot analysis of viable

671 CD45⁺CD45RA⁻TCR⁺CD4⁺CD45RC^{high or low/-} mononuclear leukocytes from muscle (left
672 panels) or spleen (right panels) of WT or *Dmd*^{mdx} rats from animals at 12 weeks of age. **E)**
673 Absolute numbers of CD45⁺CD45RA⁻TCR⁺CD4⁺CD45RC^{high} cells in muscle of spleen of WT
674 or *Dmd*^{md} rats during time. **F)** Absolute numbers of CD45⁺TCR⁻CD45RC^{low/-} cells in muscle
675 of spleen of WT or *Dmd*^{md} rats during time. **G)** Representative dot-plot analysis of viable
676 CD45⁺TCR⁻CD45RC^{high or low/-} mononuclear leukocytes from muscle (left panels) or spleen
677 (right panels) from animals at 12 weeks of age. **H)** Absolute numbers of CD45⁺TCR⁻
678 CD45RC^{high} cells in muscle of spleen of WT or *Dmd*^{mdx} rats during time.

679

680

681 **Supplementary figure 4. Effects of anti-CD45RC or prednisolone treatments on muscle**

682 **fibrosis and serum CK levels.** Littermate wild-type (WT) or *Dmd*^{mdx} (KO) rats were treated

683 with anti-CD45RC or prednisolone since week 2 of age. **A)** *Biceps femoris* muscles were

684 harvested at 12 weeks of age, fixed and paraffin embedded, connective/fibrotic tissue was

685 stained with picrosirius for connective tissue and the stained surface was quantified and

686 expressed as the percentage of total area of the tissue analyzed (47 mm²). WT isotype control,

687 n=6; WT anti-CD45RC, n=3; KO isotype control, n=6; KO anti-CD45RC, n=3; KO

688 prednisolone, n=6. * p<0.05 vs. WT controls and anti-CD45RC-treated animals. **B)**

689 Representative picrosirius (purple) staining for animals of the indicated group treatments. **C)**

690 (left panel) Sera of *Dmd*^{mdx} and WT rats treated with prednisolone or vehicle (NaCl) were

691 collected at 12 weeks of age and CK levels were determined simultaneously in all samples.

692 WT NaCl, n=3; WT prednisolone, n=3; KO NaCl, n=5; KO prednisolone, n=6. (right panel)

693 Sera of *Dmd*^{mdx} and WT rats treated with anti CD45RC or isotype control were collected at 4,

694 8 12 and 16 weeks of age and CK levels were determined simultaneously in all samples. WT

695 isotype control (n=12, 4, 11, 4 at 4, 8, 12 and 16 weeks of age, respectively); WT anti

696 CD45RC (n=13, 8, 11, 3 at 4, 8, 12 and 16 weeks of age, respectively); KO isotype control
697 (n= 6, 4, 14, 3 at 4, 8, 12 and 16 weeks of age, respectively); KO anti CD45RC (n=8, 5, 13, 4
698 at 4, 8, 12 and 16 weeks of age, respectively).

699

700 **Supplementary figure 5. Cytofluorimetry analyses of CD45RC expression in blood T**
701 **cells of DMD patients and controls.** Human peripheral blood was drawn, red blood cells
702 were lysed and white blood cells were incubated with a viability dye and MAbs directly
703 coupled with the indicated fluorochromes defining CD3, CD4, CD8 and CD45RC or isotype
704 controls followed by cytofluorimetry analyzes. Ordinate depict reactivity with anti-CD45RC
705 MAb or isotype control and the boxes define CD45RC^{high} or CD45RC^{low/-} cells. Abscissa
706 depict reactivity with anti-CD4 or CD8 MAbs among CD3⁺ cells. Controls were young
707 patients (6-17 years-old) comparable in age to DMD patients and that were hospitalized for
708 pathologies not involving the immune or the neuromuscular systems.

709

710 **Supplementary figure 6. Cytofluorimetry analyses of CD45RC expression in blood non-**
711 **T cells of DMD patients and controls.** Human peripheral blood was drawn, red blood cells
712 were lysed and white blood cells were incubated with a viability dye and MAbs directly
713 coupled with the indicated fluorochromes defining CD14⁺ monocytes, CD19⁺ B cells,
714 CD16+56⁺ NK cells and CD45RC^{high} cells or isotype controls followed by cytofluorimetry
715 analyzes. Only one DMD and one control patients are showed as representative example.
716 Ordinate depict reactivity with anti-CD45RC MAb or isotype control and the boxes define
717 CD45RC^{high} cells. Abscissa depict reactivity with anti-CD14, anti-CD19 or anti-CD16+56
718 MAbs. Controls were young patients (6-17 years-old) comparable in age to DMD patients and
719 that were hospitalized for pathologies not involving the immune or the neuromuscular
720 systems.

721

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838

839

840

841

842 **Table 1**

843 Primer sequences

Primer name	Sequence	844
Arg-1 F	CCA ACTCTTGGGAAGACACCA	
Arg-1 R	GTGATGCCCCAGATGACTTT	
iNOS F	GACCAA ACTGTGTGCCTGGA	
iNOS R	TACTCTGAGGGCTGACACAAGG	
HO-1 F	CCACAGCTCGACAGCATGTC	
HO-1 R	GTTTCGCTCTATCTCCTCTTCCA	
IFN γ F	AGTGTCATCGAATCGCACCTG	
IFN γ R	TTCTGGTGACAGCTGGTGAAT	
IL6 F	GCAAGAGACTTCCAGCCAGTT	
IL6 R	CATCATCGCTGTTCATAACAATCA	
TNF α F	CTTCTCATTCTGCTCGTGG	
TNF α R	GCTACGGGCTTGTCACCTCG	
TGF β F	CTCAACACCTGCACAGCTCC	
TGF β R	ACGATCATGTTGGACA ACTGCT	
IL-10 F	TGCTATGTTGCCTGCTCTTACTG	
IL-10R	TCAAATGCTCCTTGATTCTGG	
IL-34 F	CTGGCTGTCCTCTACCCTGA	
IL-34 R	TGTCGTGGCAAGATATGGCAA	
Areg F	AGATCGCGTTAGCAGCCATAA	
Areg R	TCAGCTAGGCTATGGCATGTG	
rIl-1b Fw2	ACCTGTCCTGTGTGATGAAAGACG	
rIl-1b Rev2	CTGCTTGAGAGGTGCTGATG	
IDO F	GCTGCCTCCCATTCTGTCTT	
IDO R	TGCGATTTCCACCATTAGAGAG	
Rln3 F	CTGCGGTCGGGAGTTCATC	
RIN3 R	CCAGGTGGTCTGTATTGGCTT	
rRLN3-Fw2	GACATCTTGGCCCACGACCCTCT	
rRLN3-Rev2	CTCTGCTGCCCCGAACCACTCCG	

Figure 1. Number of leucocytes in skeletal muscles and spleens of *Dmd^{mdx}* rats.

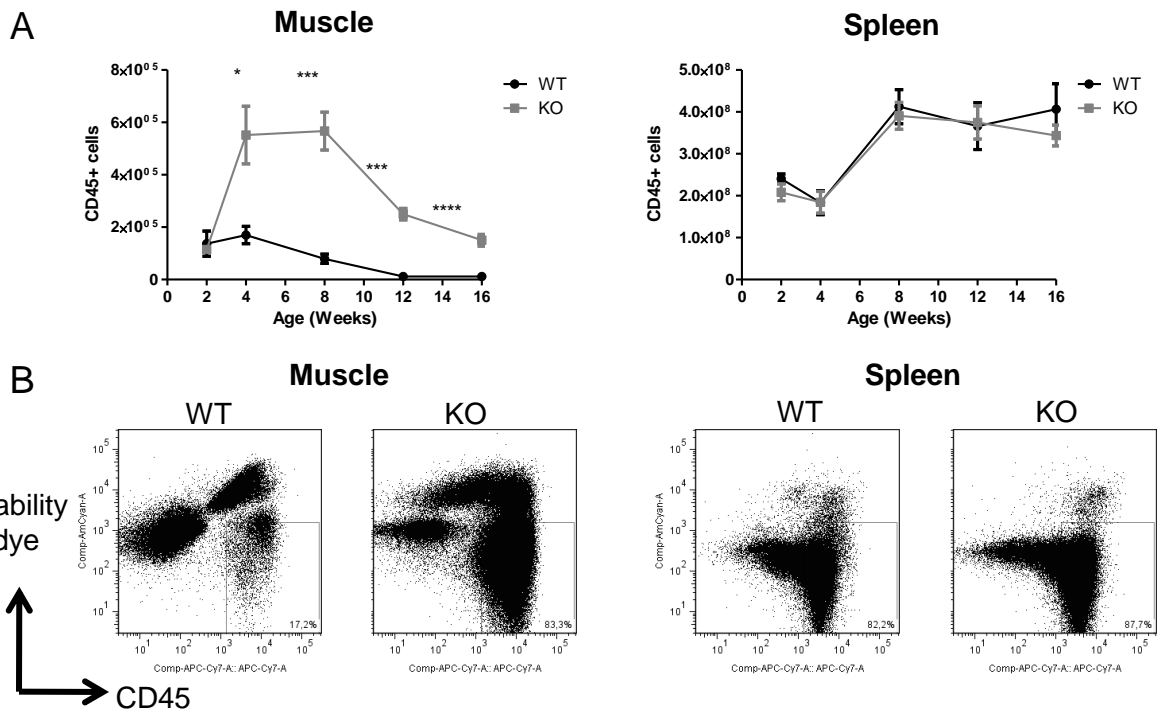


Figure 2. Macrophages in skeletal muscles and spleens of *Dmd^{mdx}* rats.

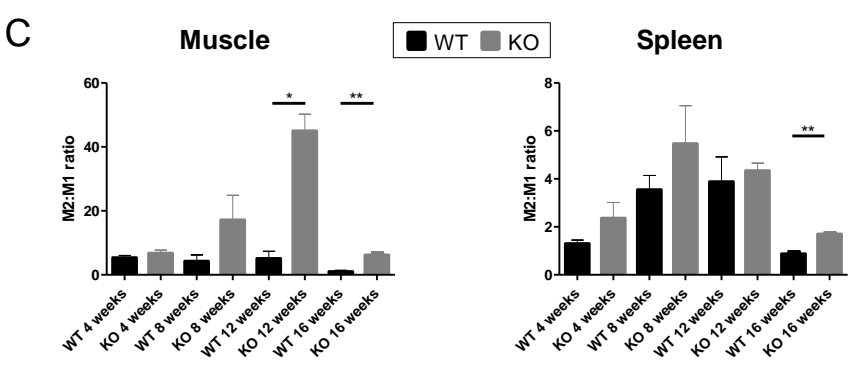
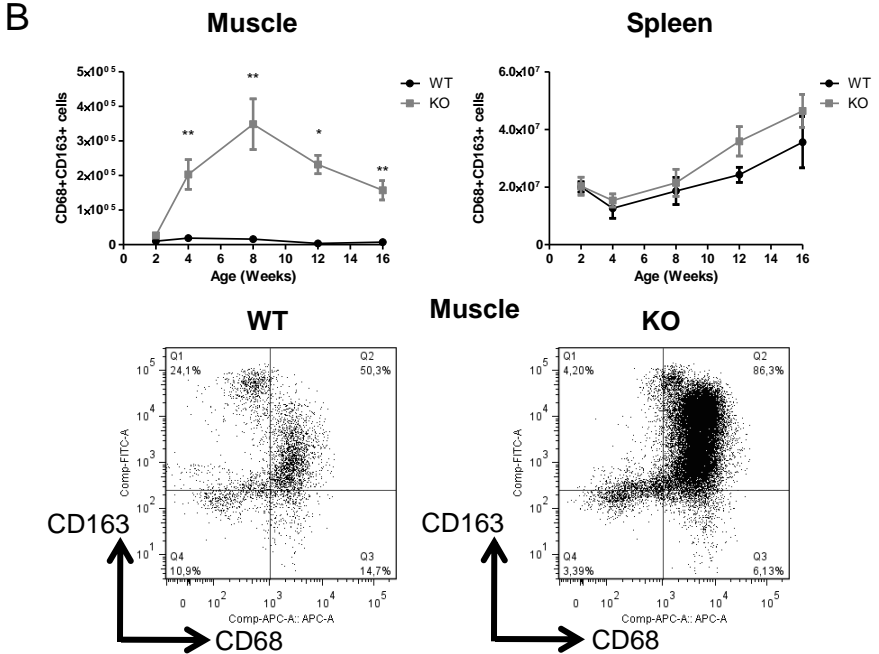
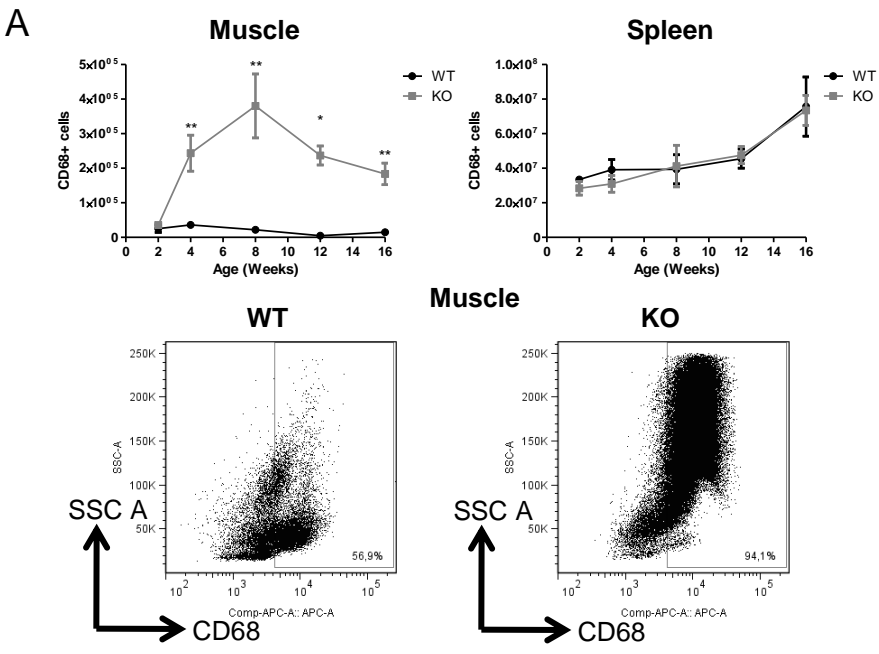


Figure 3. T cells in skeletal muscle and spleens of *Dmd^{mdx}* rats.

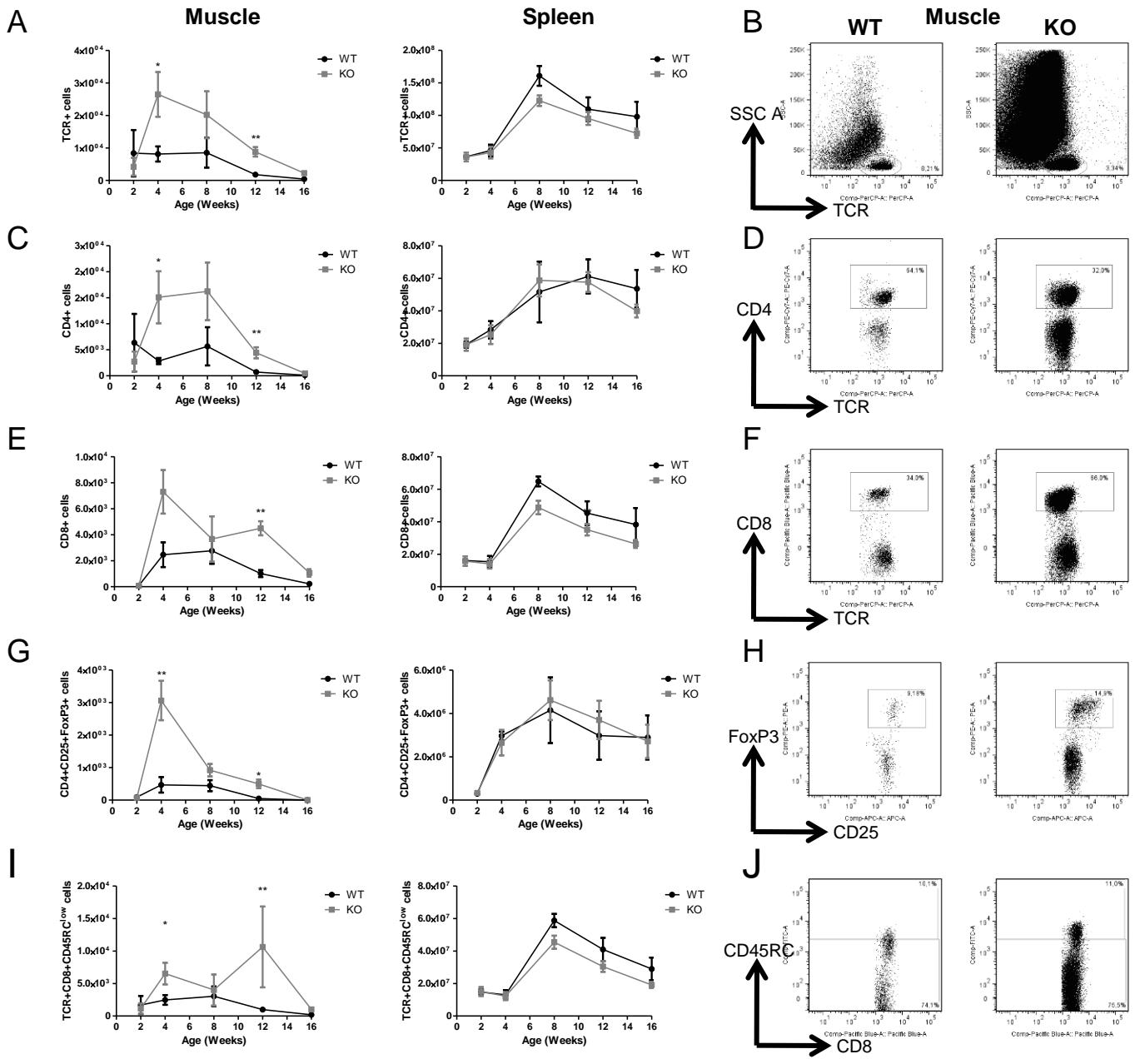


Figure 4. Immunohistochemical detection of leukocytes in skeletal and cardiac muscles of *Dmd^{mdx}* rats.

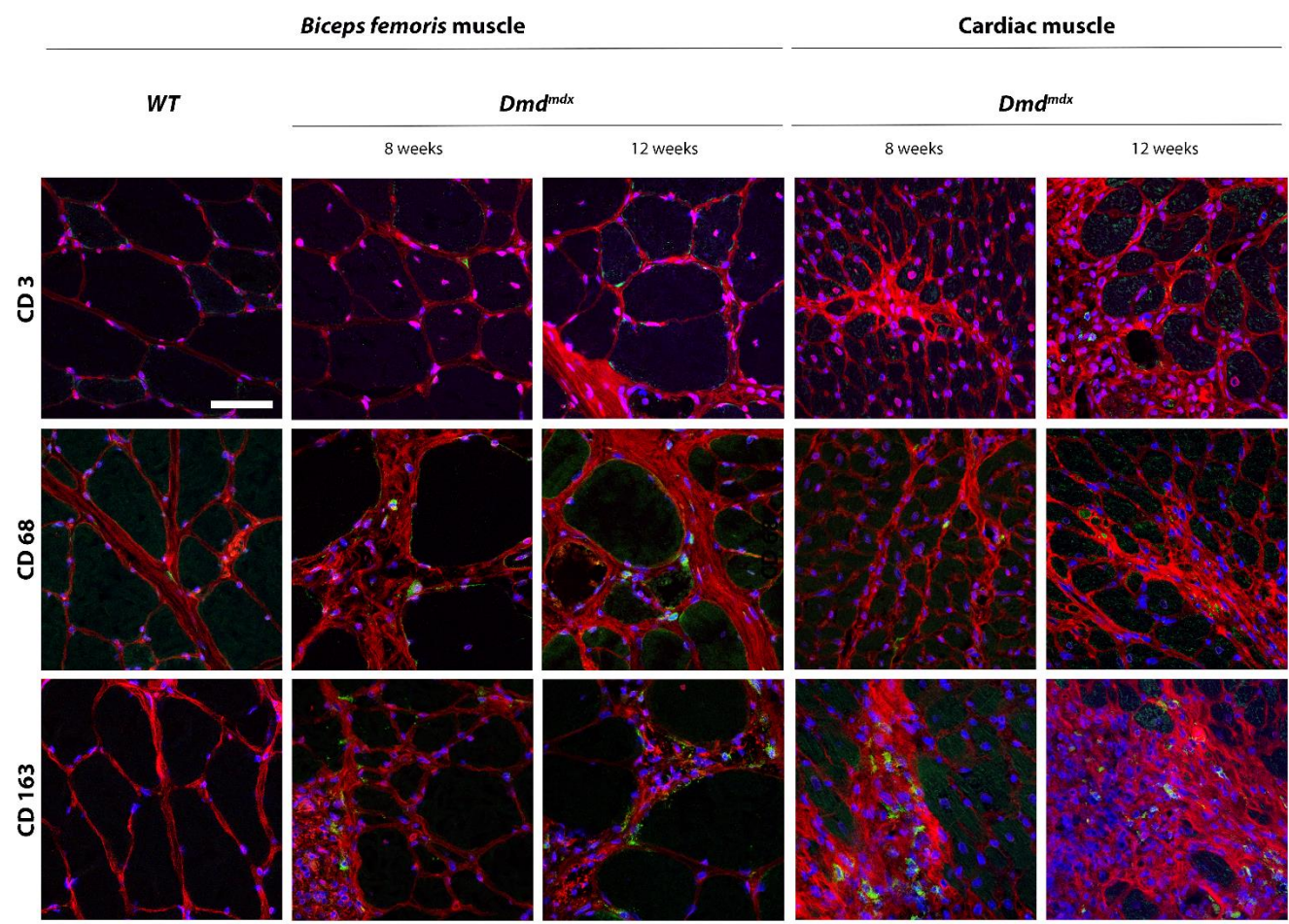
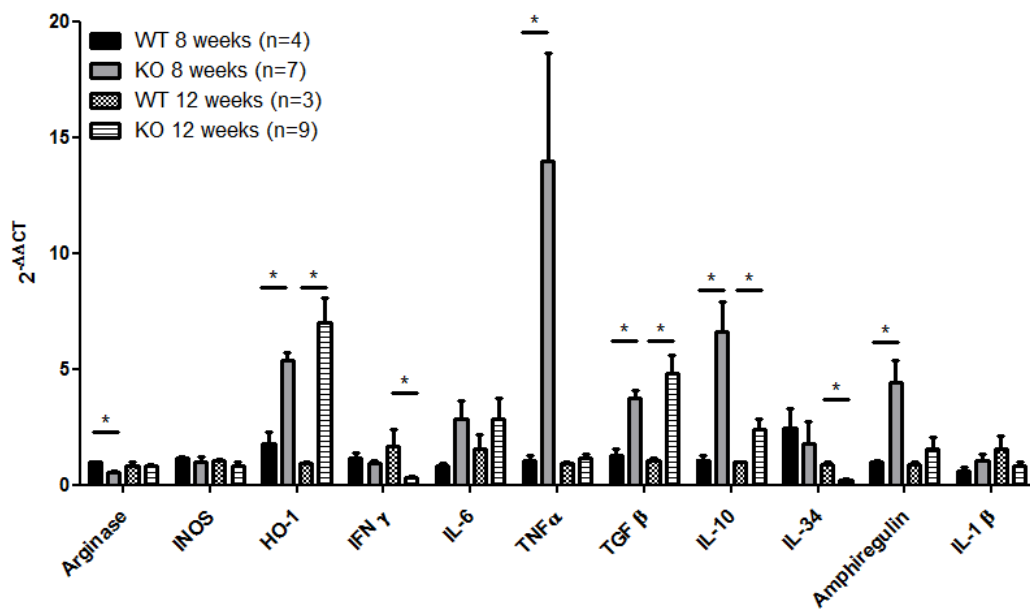


Figure 5. Inflammation markers and growth factors in skeletal muscles of *Dmd^{mdx}* rats.

A



B

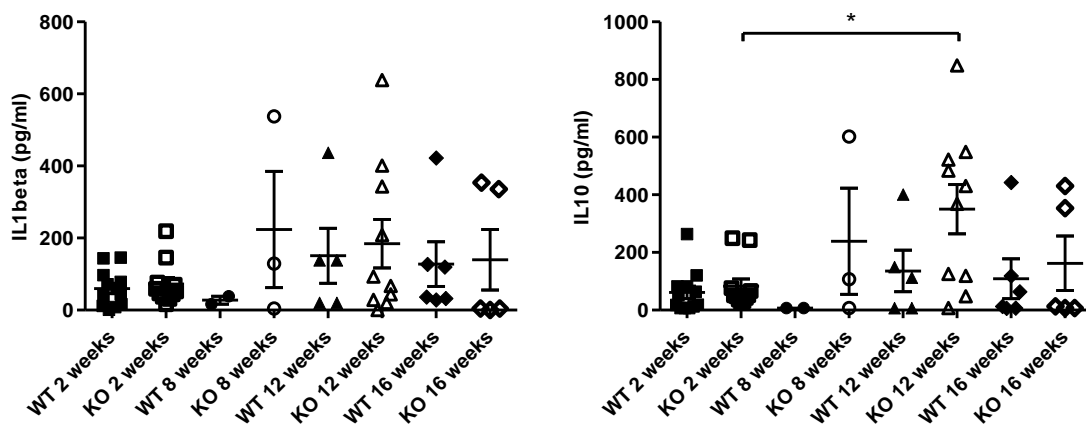


Figure 6. Effect of treatment with anti-CD45RC on lymphoid cell populations, forelimb muscle strength and animal growth.

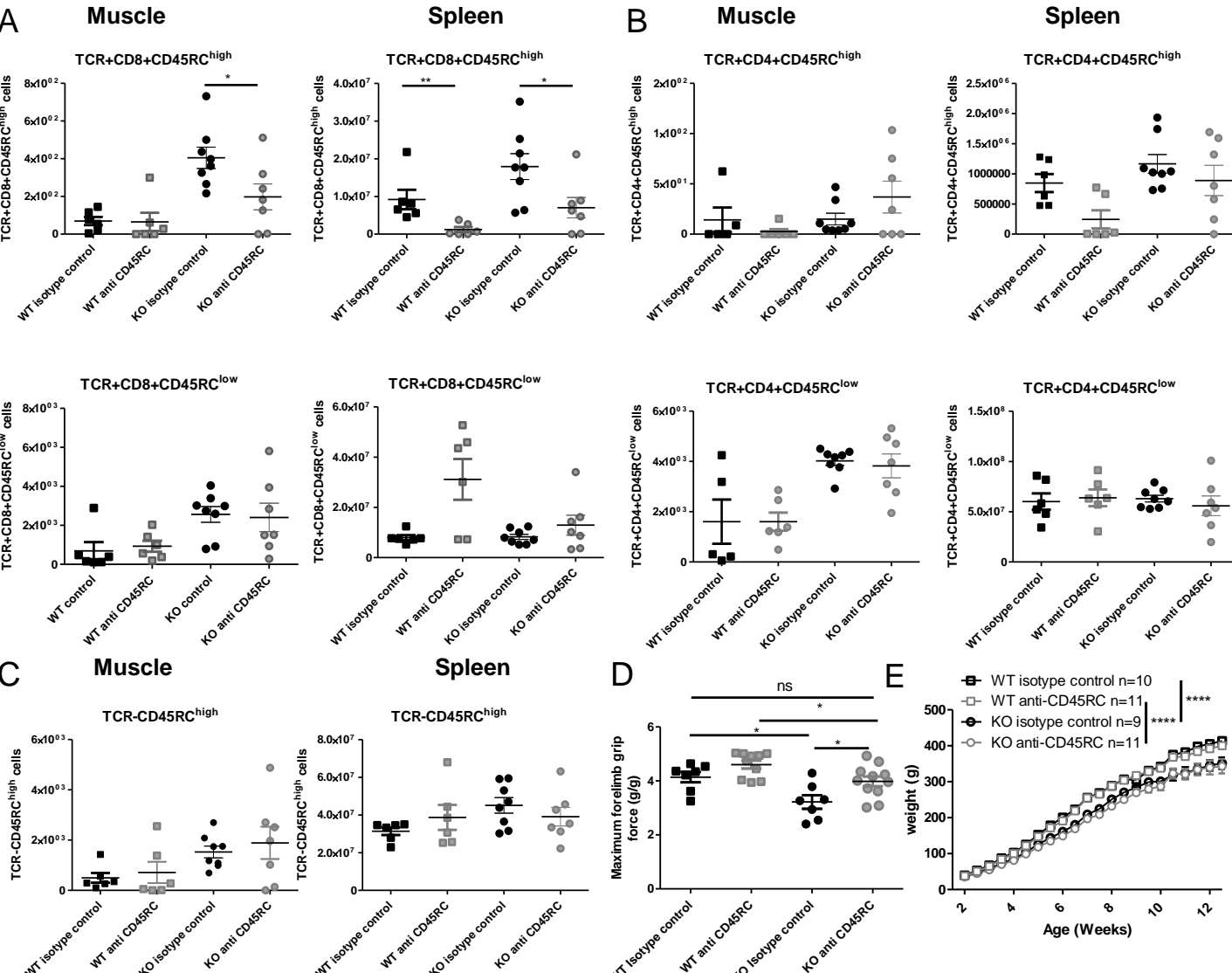


Figure 7. Treatment with prednisolone on lymphoid cell populations and forelimb muscle strength.

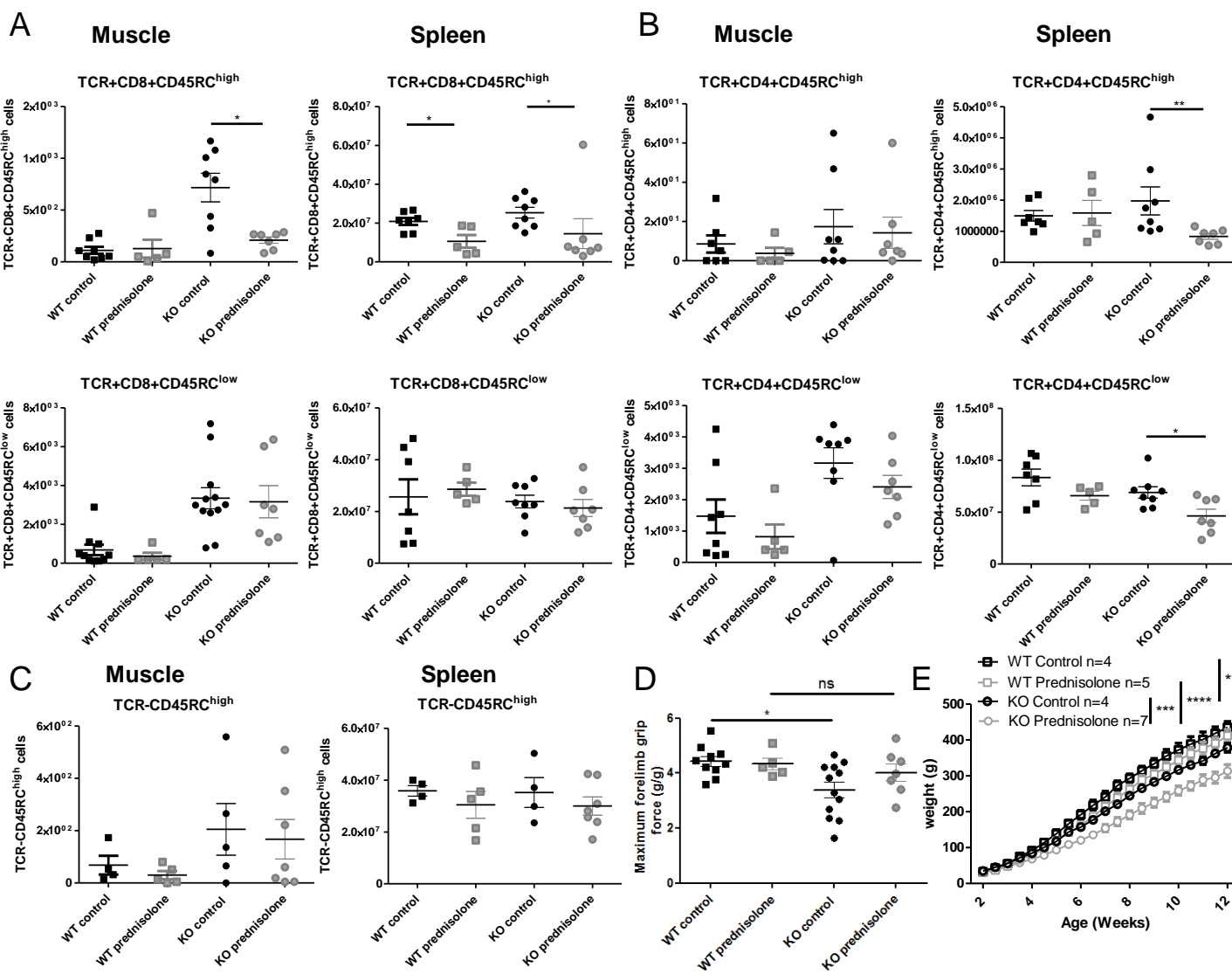


Figure 8. CD45RC⁺ cells in rat and human dystrophin-deficient skeletal muscles.

