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Immunological characterization of dystrophin-deficient *Dmd^{mdx}* rats.

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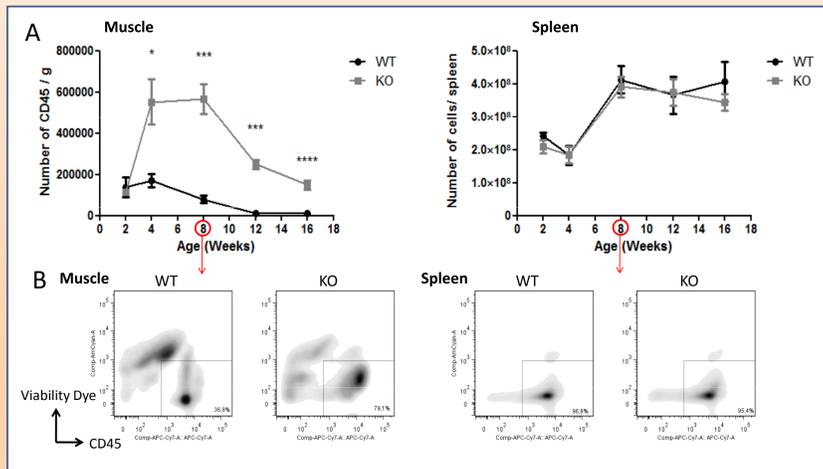
§Equal contribution

Introduction

Duchenne Muscular Dystrophy (DMD) is a severe genetic muscle-wasting disorder due to the lack of dystrophin characterized by a progressive muscle weakness and a cardiomyopathy leading to premature death. The dystrophin-deficient *Dmd^{mdx}* rats were generated using TALENs and offer a more reliable representation of human DMD, with marked muscle strength reduction, cardiomyopathy and muscle fibrosis that are higher than those observed in the *mdx* mouse model (1). A role for inflammation and autoimmune responses in muscle damages was shown both in DMD patients and the *mdx* mouse model (2).

In this study, we assessed by flow cytometry and immunohistochemistry the immune cell subsets infiltrating *Dmd^{mdx}* rat skeletal and cardiac muscles especially immunoregulatory and pro-inflammatory subsets (M1 and M2 macrophages, CD4⁺ and CD8⁺ Teff or Tregs...).

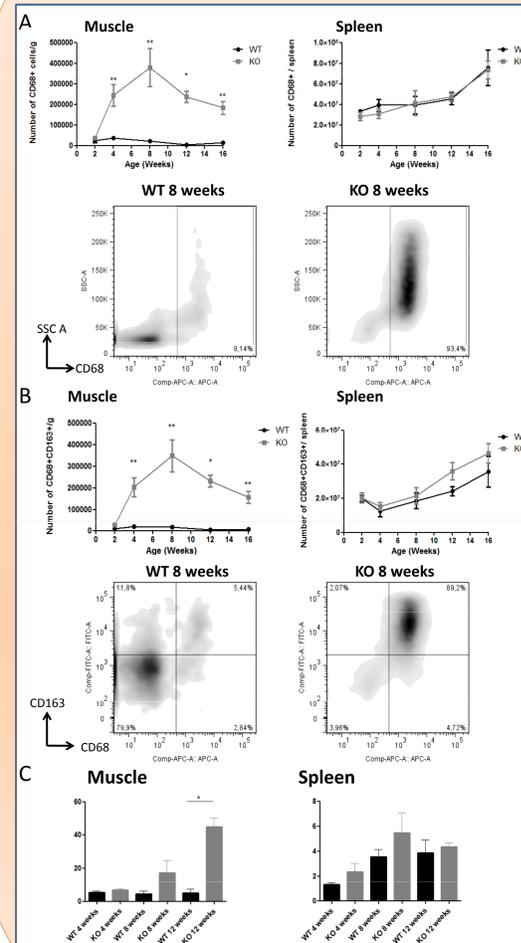
1. Leukocytes in muscle and spleen of *Dmd^{mdx}* rats.



(A) Total number of CD45⁺ leukocytes in spleen (right panel) or number of CD45⁺ leukocytes per gram of muscle (left panel). (B) Representative dot plots of WT or *Dmd^{mdx}* 8 weeks old rat muscle (left) or spleen (right) single-cell suspension showing gating on viable and CD45 positive cells.

Muscle leukocytes were low at 2 weeks of age and comparable to WT littermates. **Leukocyte infiltration in muscle of *Dmd^{mdx}* rats was maximal at 4 and 8 weeks, decreasing at 12 and 16 weeks at levels statistically higher than those observed in WT littermates (Fig. 1A).** No statistical difference was seen in spleen of WT and *Dmd^{mdx}* rats (Fig. 1B).

2. Macrophages in skeletal muscle of *Dmd^{mdx}* rats.



In *Dmd^{mdx}* rats, macrophages represented >90% of muscle leukocytes. (Fig. 2A).

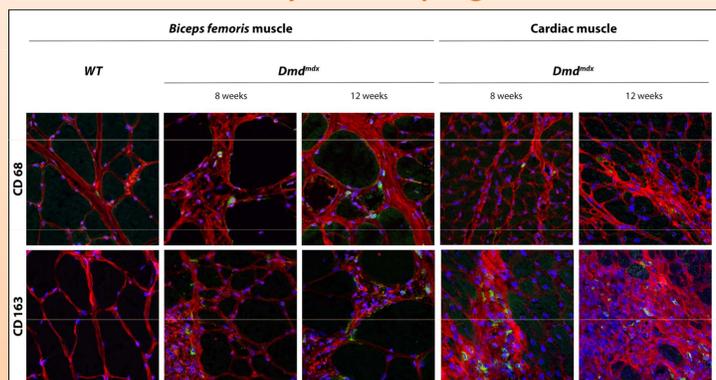
In muscles of *Dmd^{mdx}* rats at 12 weeks there was 8 times more M2 than M1 macrophages (Fig. 2C).

Fibrosis in *Dmd^{mdx}* rat's muscle begins at week 8 and is maximal at week 12 (data not shown). Analysis by RT-qPCR of arginase expression by muscle infiltrating leukocytes is under way.

In *mdx* mice, in early stages of the pathology a majority of leukocytes are M1 macrophages. At later stages M2 macrophages predominate, inhibit M1 macrophages, promote myofiber regeneration and later promote fibrosis by liberation of arginase (3).

(A) Total number of CD68⁺ cells in spleen (upper right panel) or number of CD68⁺ cells per gram of muscle (upper left panel). Representative dot plots of WT or *Dmd^{mdx}* 8 weeks old rat muscle single-cell suspension showing gating on CD68⁺ cells (lower panel). (B) Total number of CD68⁺ cells per gram of muscle (upper right panel) or number of CD68⁺ cells per gram of muscle (upper left panel). Representative dot plots of WT or *Dmd^{mdx}* 8 weeks old rat muscle single-cell suspension showing gating on CD68⁺ cells (lower panel). (C) Macrophages type 1 (TCR-CD68⁺CD163⁻ cells) or spleen (right panel) or spleen (right panel) of WT or *Dmd^{mdx}* rats.

3. Immunohistochemistry of macrophages in *Dmd^{mdx}* muscle.

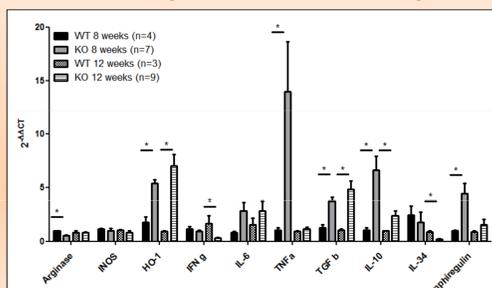


We confirmed by immunohistochemistry the infiltration of CD68⁺ and CD163⁺ leukocytes in *Dmd^{mdx}* rat biceps femoris muscle compare to WT muscle.

We observed CD68⁺ and CD163⁺ cells infiltration in cardiac muscle of *Dmd^{mdx}* rats, showing that inflammation also occurred in cardiac muscle with an important macrophagic infiltration.

By immunochemistry we could observed that fibrosis begun at week 8 and was maximal at week 12 and thereafter, consistent with M2 macrophages infiltration.

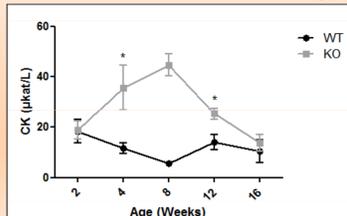
5. Analysis of inflammatory and growth factors in leukocytes.



Dmd^{mdx} and WT rats at 8 and 12 weeks of age showed that HO-1, IFN γ , TGF β , IL-10 and amphiregulin were increased in muscles of *Dmd^{mdx}* vs. WT rats at 8 and/or 12 weeks and TNF α was particularly increased at 8 weeks. Arginase and IL-34 were decreased in the muscle of *Dmd^{mdx}* rats vs. WT rats, at weeks 8 and 12 respectively.

Analysis of inflammatory and growth factors in muscles leukocytes by RT-qPCR

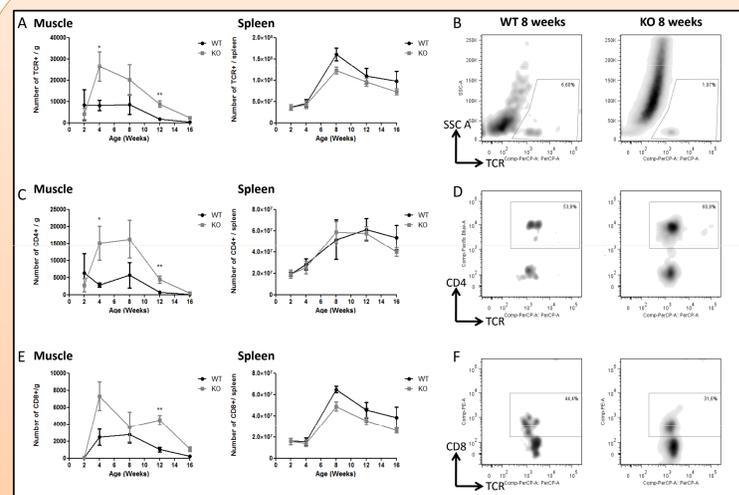
6. Inflammatory markers in sera.



Creatine kinase was increased at weeks 4 and 8 and decreased at week 12 and thereafter. This indicates muscle cell lysis.

Mesure of Creatine Kinase in sera of WT or *Dmd^{mdx}* rats

4. T cells in skeletal muscle of *Dmd^{mdx}* rats.



In *Dmd^{mdx}* rats, **T cells represented the second predominant population of muscle-infiltrating immune cells.** Infiltration of TCR⁺ cells was observed at 4 weeks, then decreased at 8 and 12 weeks. At 16 weeks there was no difference between WT and *Dmd^{mdx}* rats. A similar profile was observed for CD4⁺ and CD8⁺ cells. There was no statistical difference between number of total TCR⁺, CD4⁺ or CD8⁺ cells in spleen of WT and *Dmd^{mdx}* rats.

We also observed an increase of CD4⁺ Treg cells (CD4⁺Foxp3⁺CD25⁺) in *Dmd^{mdx}* rat muscle at 4 and 12 weeks (data not shown).

An important role of T cell population in the pathology development has been shown in *mdx* mice (3). This study showed an increase in CD3⁺, CD4⁺ and CD4⁺FoxP3⁺ cells in *mdx* mice muscle compared to WT and a protective role for CD4⁺ Tregs in this pathology.

Conclusion

In this study, we phenotyped by flow cytometry and immunohistochemistry the immune cell subsets infiltrating *Dmd^{mdx}* rat skeletal and cardiac muscles especially immunoregulatory and proinflammatory subsets (M1 and M2 macrophages, CD4⁺ and CD8⁺ Teff or Tregs). Infiltration level was absent or very low at 2 weeks of age, peaked at 4 and 8 weeks and decreased at 12 weeks. **Macrophages with M2 profiles represent the majority (>90%) of infiltrating immune cells and Teff cells were the majority of the remaining ones.** We also analyzed muscle enzymes and cytokines in sera. Creatine kinase was increased at weeks 4 and 8 and decreased at week 12 and thereafter. This results are consistent with those observed in *mdx* mice model.

Experiments are ongoing to investigate the possibility of reducing disease in *Dmd^{mdx}* rats by administrating different immunomodulatory treatments.