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ARTICLE

Comparative transcriptome analysis of muscular dystrophy models *Large*^{myd}, *Dmd*^{mdx}/*Large*^{myd} and *Dmd*^{mdx}: what makes them different?

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Muscular dystrophies (MD) are a clinically and genetically heterogeneous group of Mendelian diseases. The underlying pathophysiology and phenotypic variability in each form are much more complex, suggesting the involvement of many other genes. Thus, here we studied the whole genome expression profile in muscles from three mice models for MD, at different time points: *Dmd*^{mdx} (mutation in dystrophin gene), *Large*$^{myd-/-}$ (mutation in *Large*) and *Dmd*^{mdx}/*Large*$^{myd-/-}$ (both mutations). The identification of altered biological functions can contribute to understand diseases and to find prognostic biomarkers and points for therapeutic intervention. We identified a substantial number of differentially expressed genes (DEGs) in each model, reflecting diseases' complexity. The main biological process affected in the three strains was immune system, accounting for the majority of enriched functional categories, followed by degeneration/regeneration and extracellular matrix remodeling processes. The most notable differences were in 21-day-old *Dmd*^{mdx}, with a high proportion of DEGs related to its regenerative capacity. A higher number of positive embryonic myosin heavy chain (eMyHC) fibers confirmed this. The new *Dmd*^{mdx}/*Large*$^{myd-/-}$ model did not show a highly different transcriptome from the parental lineages, with a profile closer to *Large*$^{myd-/-}$, but not bearing the same regenerative potential as *Dmd*^{mdx}. This is the first report about transcriptome profile of a mouse model for congenital MD and *Dmd*^{mdx}/*Large*^{myd}. By comparing the studied profiles, we conclude that alterations in biological functions due to the dystrophic process are very similar, and that the intense regeneration in *Dmd*^{mdx} involves a large number of activated genes, not differentially expressed in the other two strains.
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INTRODUCTION

Muscular dystrophies (MD) include various forms of inherited disorders with monogenic etiologies, characterized by progressive muscular degeneration and weakness.[1] Duchenne muscular dystrophy (DMD) is the most common form, affecting one in 5000 males. The disease is caused by mutations in the dystrophin gene that lead to total absence of the protein in muscle and consequent muscle degradation.[2] Dystrophin is responsible for the connection between cytoskeleton and plasma membrane, providing mechanical stability. *Dmd*^{mdx} mouse is an animal model for DMD, presenting a mutation that also results in the absence of dystrophin.[3] The muscles of these mice show many features of a dystrophic muscle, including degeneration and fibrosis; nevertheless, differently from DMD patients, they have a significant muscle regeneration and an almost normal phenotype; thus, *Dmd*^{mdx} is good for genetic and biochemical studies, but not for clinical trials.[4]

Congenital muscular dystrophies (CMD) compose a subgroup of MD, including forms caused by glycosylation defects in the α-dystroglycan protein, a member of the dystrophin–glycoprotein complex (DGC).[5] Glycosylation of α-dystroglycan is crucial for its binding to laminin in the extracellular matrix (ECM), and it is a process performed by a series of glycosyltransferase enzymes, including LARGE.[6] Mutations in human *LARGE* gene cause a severe form of congenital muscular dystrophy[7] and a deletion in the murine *Large* gene causes a similar phenotype in the *Large*^{myd} mice.[8]

To evaluate the effect of α-dystroglycan hypoglycosylation in the modulation of the dystrophic phenotype and the impact of the double absence of dystrophin and LARGE for the assembly of DGA complex, we developed in our laboratory a double-mutant mouse for these two proteins. The *Dmd*^{mdx}/*Large*$^{myd-/-}$ animals are viable, but have a reduced life expectancy and a severe phenotype, even worse than *Large*$^{myd-/-}$ mice.[9]

Besides the fact that causative gene mutations causing muscular dystrophies have been already identified, the underlying pathophysiological pathways and phenotypic variability in each MD form are much more complex, suggesting the involvement of many other genes. Thus, studying the whole genome expression profile might significantly contribute to identifying altered biological functions that could lead to a better understanding of the disease, including identifying possible prognostic biomarkers and points for therapeutic intervention.

MATERIALS AND METHODS

Animals and sample preparation

The experimental procedures were approved by the Ethics Commission for the Use of Animals from Biosciences Institute (CEUA/IBUSP; Protocol 200/2014).

The mice were kept under appropriated controlled conditions, with water and food *ad libitum*, in the animal house of Human Genome and Stem Cell Research Center.

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Four mouse strains were used:

- C57BL/10ScSn-*Dmd*^{mdx}/J, named here as *Dmd*^{mdx}, a model for DMD, bearing a point mutation in exon 23 of dystrophin gene.[3]
- B6C3Fe *a/a-Large*^{myd}/J, named here as *Large*$^{myd-/-}$, has an intragenic deletion in *Large* gene and is the model for dystroglycanopathy.[8] *Large*$^{myd-/-}$ mice show abnormal skeletal muscle fiber morphology, decreased body weight, postnatal growth delay, reduced fertility and life expectancy.
- *Dmd*^{mdx}/*Large*$^{myd-/-}$, double-mutant created in our laboratory, bearing mutation in both dystrophin and LARGE genes. The *Dmd*^{mdx}/*Large*$^{myd-/-}$ animals are very severely affected. Details about crossing are provided in our previous article.[9]
- C57BL/6 J – wild-type mice.

A total of 60 mice were studied: five animals of each lineage – *C57BL*, *Dmd*^{mdx}, *Large*$^{myd-/-}$, *Dmd*^{mdx}/*Large*$^{myd-/-}$ – of three different ages – 3 weeks old, 3 months and 6 months old. The calf muscles were dissected and rapidly frozen in liquid nitrogen. Total RNA was extracted using the RNeasy Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Quantity and quality of RNA was assessed with Nanodrop 8000 Spectrophotometer (Thermo Scientific, Schwerte, Germany), agarose gel electrophoresis and integrity with Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). Only samples with 260/280 ratio greater than 1.8, preserved rRNA 28S/18S ratio and RIN (RNA integrity number) >7.0 were used.

Microarray hybridization, data processing and mining

Expression measurements were performed with Affymetrix Mouse Gene 1.0 ST arrays, according to the manufacturer's recommendations. Quality control analysis was done with the Expression Console Software (Affymetrix, Santa Clara, CA, USA), which also provides gene expression values using Robust Multi-array Average (RMA) pre-processing method. Screening of differentially expressed genes (DEGs) was performed in the MeV (MultiExperiment Viewer) software, using SAM algorithm.[10] We selected as significantly DEGs those transcripts whose false discovery rate (FDR) values were lower than 0.05. Raw data have been deposited at Gene Expression Omnibus (GEO) database (accession number GSE72151).

Transcriptome analysis

Gene annotation was performed in IPA (http://www.ingenuity.com). KEGG pathways (Kyoto Encyclopedia of Genes and Genomes, www.genome.jp/kegg) enrichment analysis was done with EXPANDER (http://acgt.cs.tau.ac.il/expander/). Gene Ontology Biological Process (GO) (The Gene Ontology Consortium, www.geneontology.org) enrichment analysis was performed with DAVID web tool (https://david.ncifcrf.gov/) to calculate the representation of biological functions.

qRT-PCR

To obtain cDNA, 1 μg of RNA was retro-transcribed with MMLV enzyme (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. For qPCR, we used the protocol described by Gosselin *et al.*[11] Amplification was done with SybrGreen Master Mix (Roche, Vilvoorde, Belgium). The run was performed in the 7500 Applied Biosystems thermocycler (Applied Biosystems, Foster City, CA, USA). Results were analyzed with 7500 Software v2.0.6 (Applied Biosystems). Fold changes of the target genes were calculated as mean values of $2^{-\Delta\Delta CT}$.

Antibodies and immunofluorescence

Muscle cryosections (6 μm thick) were labeled with primary antibodies against laminin (1/50 dilution, Abcam, Cambridge, MA, USA – Ab80580) and embryonic myosin heavy chain (eMyHC) (1/30 dilution, Vector Laboratories, Burlingame, CA, USA – VPM664). Secondary antibodies were anti-rat FITC (Sigma-Aldrich, St Louis, MO, USA – F6258) and anti-mouse Cy3 (Sigma-Aldrich – C2181). Nuclei were visualized using DAPI diluted in mounting medium Vectashield (Vector Laboratories). The sections were visualized under a conventional upright microscope (Axio Imager.Z1, Carl Zeiss, Oberkochen, Germany), and images acquired with Axion Vision Software (Carl Zeiss).

RESULTS

Differentially expressed genes

We compared each dystrophic lineage with control mice and obtained a large number of DEGs, of which the majority is upregulated (Figure 1a). The complete lists of DEGs are available at Supplementary Information (Supplementary Tables S1–S9).

Twenty-one day-old *Dmd*^{mdx} mice showed the largest number of DEGs, of which the majority (268) is not common to other ages; with aging, this number decreases (268, 3, 72). For *Large*$^{myd-/-}$ (27, 35, 156) and *Dmd*^{mdx}/*Large*$^{myd-/-}$ (22, 63, 66), the number of exclusive DEGs increases with age (Figure 1b).

The results observed in 12 genes with altered expression in the microarray assay were validated by quantitative real-time PCR, using cDNA from 3 animals of each lineage and age. We selected the following genes randomly: *Ankrd1*, *Gpnmb*, *Large*, *Mmp14*, *Mstn*, *Mup1*, *Myog*, *Nos1*, *Ostn*, *Pttg1* and *Tnnt2*. All the genes presented expression values concordant with microarray analysis (Supplementary Table S25).

DEGs that define the dystrophic process

To define which genes are deregulated in the presence of a dystrophic process, regardless of the mutated gene, we overlapped the lists of DEGs of each lineage and age. We found that 31 genes are shared by all them in the three ages (Figures 2a and 3, Supplementary Table S26). All these genes are upregulated and the majority showed similar fold-change values across the ages and lineages. These genes are mainly involved in immune system functions and degeneration/regeneration processes. Additionally, it was observed that three genes are exclusive of *Large*$^{myd-/-}$, 84 are exclusive of *Dmd*^{mdx} and only 1 gene was exclusive of *Dmd*^{mdx}/*Large*$^{myd-/-}$ (Figure 2a).

Separated by age, the genes that are deregulated in the three models showed an increase from the 21 days to 6 months. Therefore, in the last stages, there are a greater number of DEGs common to all dystrophic processes (Figure 2b).

Characterization of each dystrophic strain

To verify the biological meaning of observed changes in the expression of so many genes, the DEGs were analyzed with DAVID web tool that offers information of Gene Ontology classification identifying enriched themes. The GO functions with % FDR <20 were considered significant (the categories are listed in Supplementary Tables S10–S18).

We found significant results only for upregulated genes. For each list of DEGs, we found hundreds of GO functions; many of them were similar and correlated, so they were grouped in 10 more general categories, like *Immune System and Inflammation* and *Extracellular Matrix and Adhesion*. The distributions of these categories are represented in pie charts (Figure 4). For all lineages and ages, the most representative category is *Immune System and Inflammation*.

KEGG pathways analysis also showed that the most significant enriched pathways are related to immune system functions, corroborating GO analysis (data not shown).

As the contribution of immune system components to muscular dystrophies pathogenesis is well known, we removed this category and analyzed the distribution of the remaining ones (Figure 5).

For 21-day-old *Dmd*^{mdx}, it is remarkable the representation of functions related to cell cycle and proliferation, which was reduced but maintained at the age of 3 months. *Large*^{myd} mice at the same age showed the predominance of functions related to ECM. Curiously, the double-mutant mice do not have an enrichment of cell-cycle/proliferation functions; instead, their overexpressed genes are associated with enzymatic activity.

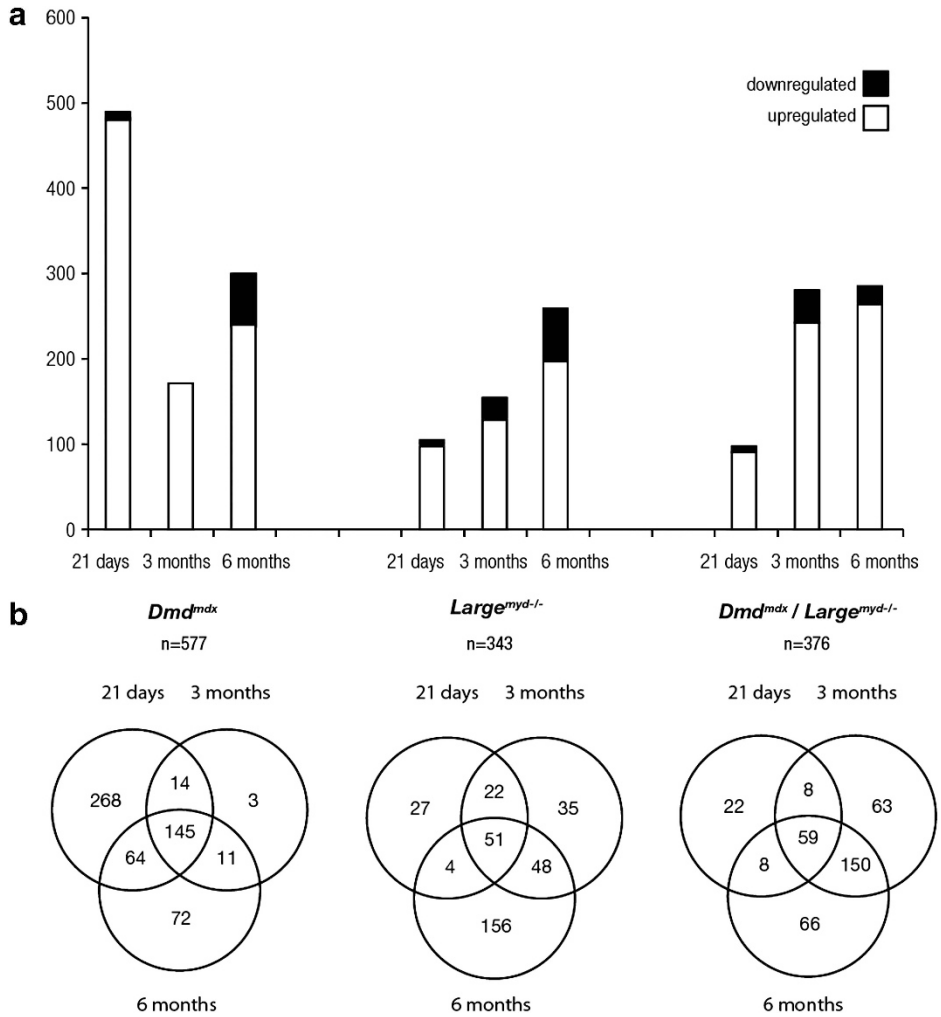

Figure 1 (**a**) Number of DEGs in the three lineages in relation to wild type. (**b**) Venn diagrams showing overlapping DEGs between three ages of each mouse lineage. The total number of genes with differential expression in the lineage was calculated considering only those with expressions values $> \pm 1.0$.

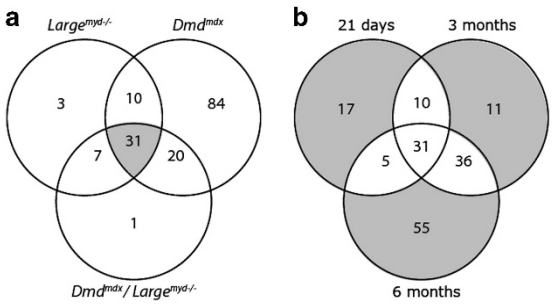

Figure 2 Venn diagrams showing overlapping DEGs between the three dystrophic models. (**a**) DEGs grouped by lineage. The gray area highlights the genes shared by all animals. (**b**) DEGs grouped by age. The grey areas highlight the genes specific of each age.

At 3 months old, for the three lineages, ECM and adhesion functions are the most representative category, followed by homeostasis. By 6 months old, the three profiles are more similar, with highlight to ECM and adhesion, and enzymatic activity functions.

Dmd^{mdx}/Large$^{myd-/-}$ versus parental strains

We also compared the double mutant with its parental strains to find which genes are deregulated between them. In general, there are few genes differentially expressed between the dystrophic mice (Table 1) (complete lists of DEGs are on Supplementary Tables S19–S24).

The 21-day-old double mutants present more downregulated genes than upregulated ones in relation to *Dmd*^{mdx}. However, among the 124 downregulated genes in this comparison, only 41 are overexpressed in the *Dmd*^{mdx}, comparing with control. In the ages of 3 and 6 months, there are less genes differentially expressed among dystrophic mice.

The overlap of lists containing the genes with differential expression during the three time points for each lineage showed that just one gene is exclusively upregulated in the *Dmd*^{mdx}/*Large*$^{myd-/-}$ mice. This gene is *Lilrb3* (*Pirb-001*) (Figure 2).

Muscle regeneration

Muscle regeneration was accessed by the staining of embryonic myosin heavy chain, in the three strains and ages. The data confirmed a reduced regeneration capacity in *Large*$^{myd-/-}$ and *Dmd*^{mdx}/*Large*$^{myd-/-}$ mice compared with *Dmd*^{mdx}. The percentages of positive fibers in each strain and age were calculated and are presented in Figure 6.

DISCUSSION

The global gene expression analysis of muscles from studied mice revealed an enormous number of genes with altered expression, some common to the three dystrophic strains, and others specific for each

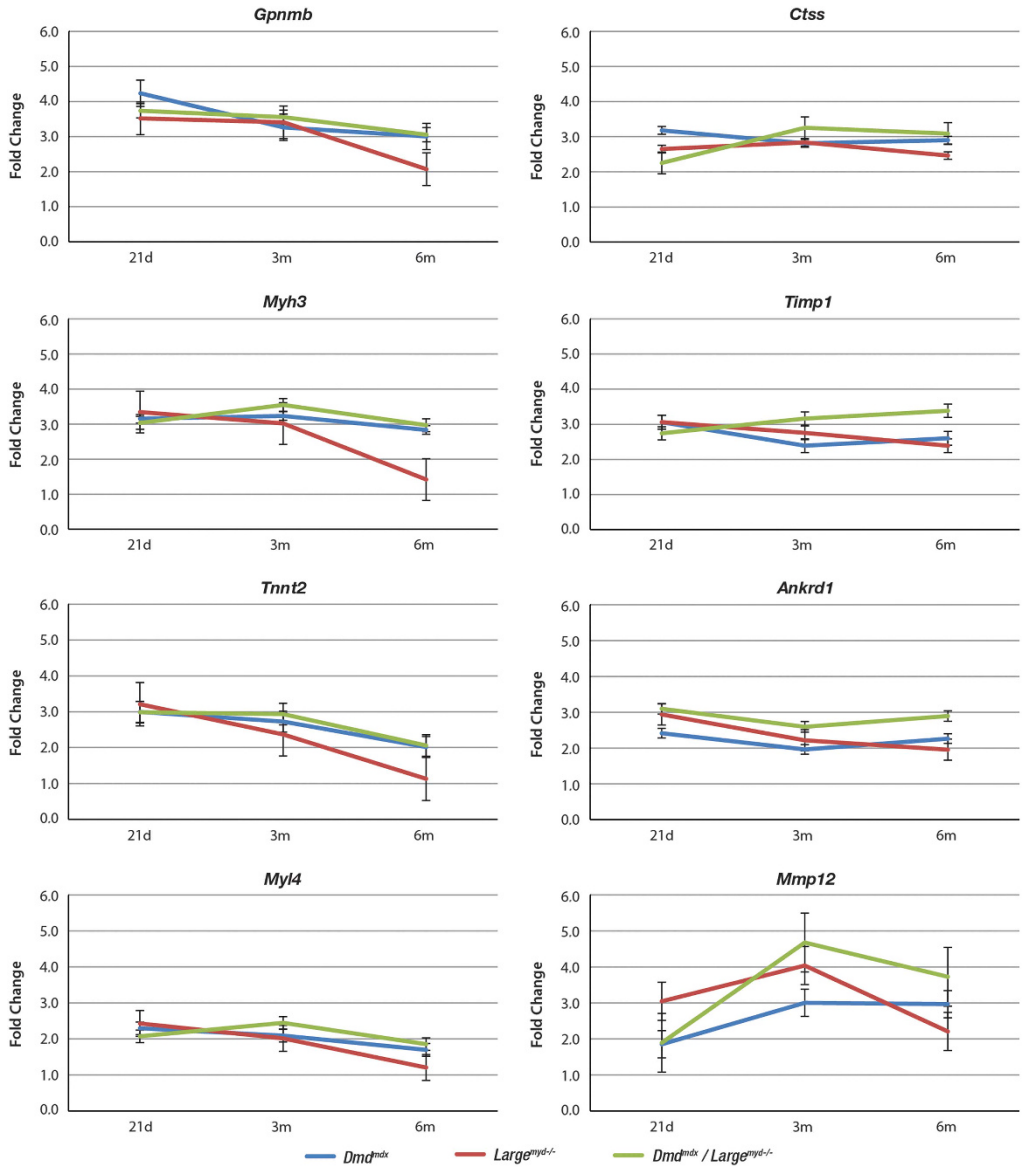

Figure 3 Fold-change values of eight selected significantly upregulated genes over time shared by the three lineages.

strain. Besides muscular dystrophies being monogenic diseases, this confirm that the effects of the mutations are much broader and complexes.

It is remarkable that the dystrophic process encompasses mainly the overexpression of many genes; only a few genes showed reduced expression values. Several transcriptome studies in *Dmd*^{mdx} mouse were published in the last years,[12–20] but no studies in *Large*$^{myd-/-}$ nor in *Dmd*^{mdx}/*Large*$^{myd-/-}$ were performed.[9] In *Dmd*^{mdx}, similar data to the observed in our study were described by Boer *et al*[14] and Porter *et al*.[17,21] Using an Affymetrix chip of about 12 000 genes/EST, Boer *et al*[14] identified 58 DEGs in *Dmd*^{mdx} limb muscles, from which 49 were unregulated while only 9 were downregulated. Porter *et al*[17,21] found a high number of differentially expressed transcripts (719) in *Dmd*^{mdx} leg muscles in different postnatal days, and the great majority was upregulated.

More upregulated genes than downregulated ones can be a reflex of the strong activation of immune response and also to high rates of protein production, due to regeneration.

The 21-day-old *Dmd*^{mdx} mice showed a higher number of DEGs than the other two lineages at the same age. This large difference can be attributed to the fact that the disease starts to develop at this age in *Dmd*^{mdx}, while the other two lineages manifest the disease from birth. At the other two time points, the number of DEGs in *Dmd*^{mdx} is closer to those seen in *Large*$^{myd-/-}$ and *Dmd*^{mdx}/*Large*$^{myd-/-}$, reflecting the stabilization of the disease process.

The effect of age in each lineage was also diverse. Although in *Dmd*^{mdx} a decrease in the number of DEGs was observed with age, in *Large*$^{myd-/-}$ and *Dmd*^{mdx}/*Large*$^{myd-/-}$, the number of DEGs increased with the progression of the disease, suggesting that in the more severe dystrophies, the disorder progression is accompanied by the upregulation of many other genes.

Characterization of the dystrophic process

We found 31 DEGs shared by all lineages and ages (Figure 2a; Supplementary Table S26) that can be considered markers of muscular dystrophy, independently of the mutation or stage of progression. These DEGs have a fold change from 1.295 to 4.676, but interestingly they were similar within each gene in the three strains.

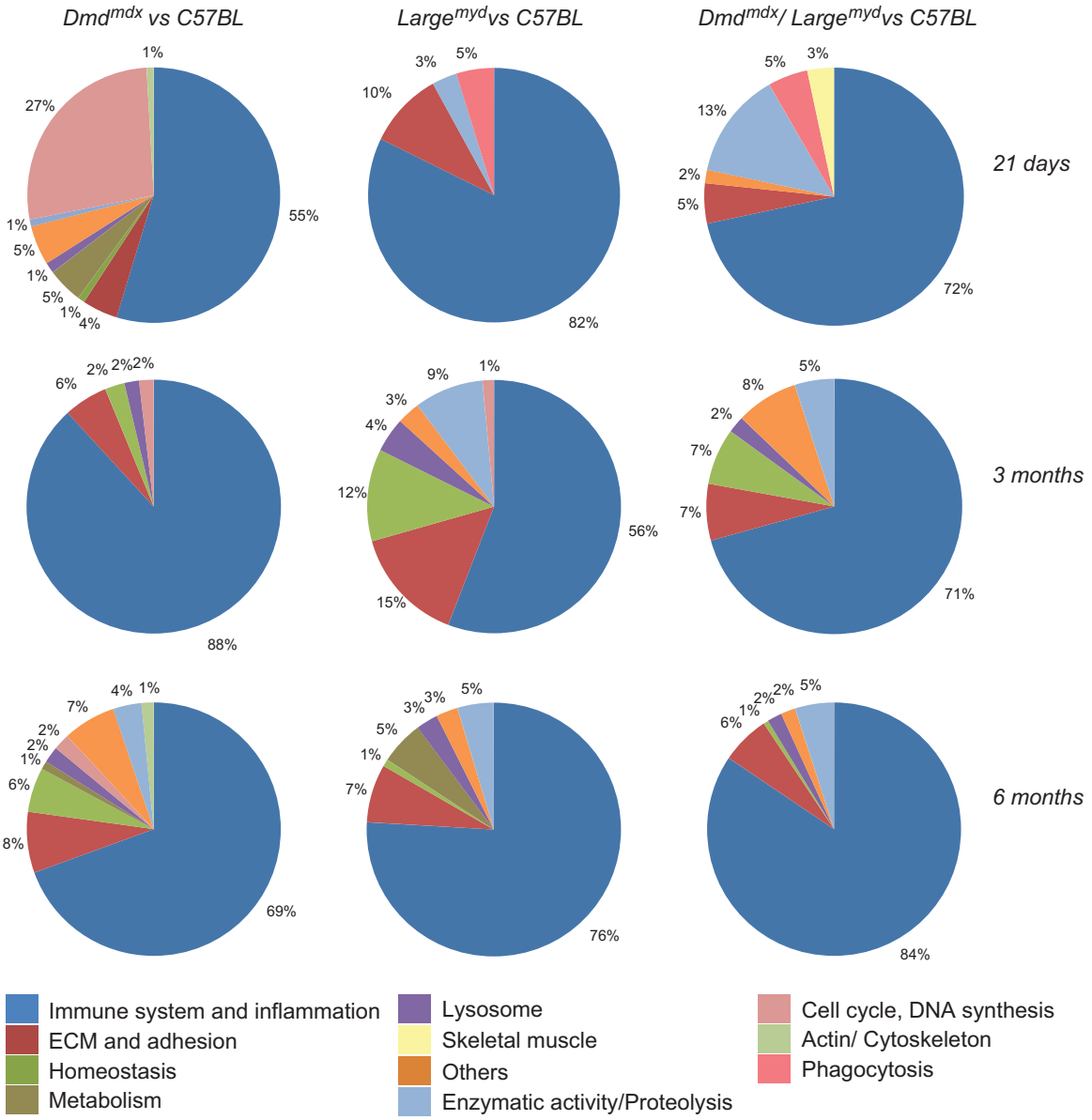

Figure 4 Enriched functional categories. Pie charts representing the enriched GO terms upregulated in all mice studied.

Of these 31 genes we highlight 8 that were already related to muscular dystrophy: *Ankrd1, Mmp12*, *Timp, Myh3*, *Myl4*, *Tnnt2*, *Ctss* and *Gpnmb* (Figure 3). With the exception of *Mmp12,* which was slightly increased in $Dmd^{mdx}$and $Dmd^{mdx}/Large^{myd-/-}$ dystrophic stains, most of these genes showed a decrease in expression with age.

Ankrd1 encodes CARP protein that participates in sarcomere stabilization.[22] It has also an important role in the transcription regulation of *Ttn* and *MyoD* genes, in the myofibril formation, cardiogenesis and myogenesis.[23] In muscle biopsies from DMD patients, CARP was suggested as a regeneration marker.[24] On the other hand, the constant expression of CARP was also considered as a marker for muscle lesion.[22] So, the role of CARP in the muscle is still dubious. Anyway, the persistent hyperexpression of *Ankrd1* in all mice strains, mainly in those severely affected, and in the latest stages of the disease, suggests a significant role of this protein in the degeneration process. In this sense, if CARP overexpression is in fact prejudicial to skeletal muscle, then its inhibition could be an interesting strategy for therapeutic intervention.[22] Nevertheless, more studies are necessary to elucidate the real role of this protein in muscular dystrophy.

Mmp12 is involved in ECM remodeling. Fibrosis is a significant feature of muscular dystrophies that sometimes correlates with the clinical picture. The matrix metaloproteinases are important regulators of the ECM, coordinating inflammation, matrix deposition and tissue reorganization.[25] Upregulation of *Mmp12* has already been reported in the Dmd^{mdx} mouse,[14] and here we are showing that the overexpression of this protein also occurs in the $Large^{myd-/-}$and $Dmd^{mdx}/Large^{myd-/-}$, suggesting a strong correlation with the dystrophic process. Given the expression of *Mmp12* in macrophages[26,27] we can propose that (1) the persistent macrophage activity would be associated with promotion of inflammation and fibrosis; (2) in opposition, this upregulation of MMP12 would be an attempt to reduce inflammation by chemokines degradation and reduction of the recruitment of more inflammatory cells. At least, both processes would be active since early stages up to the later ones and in all forms of dystrophies.

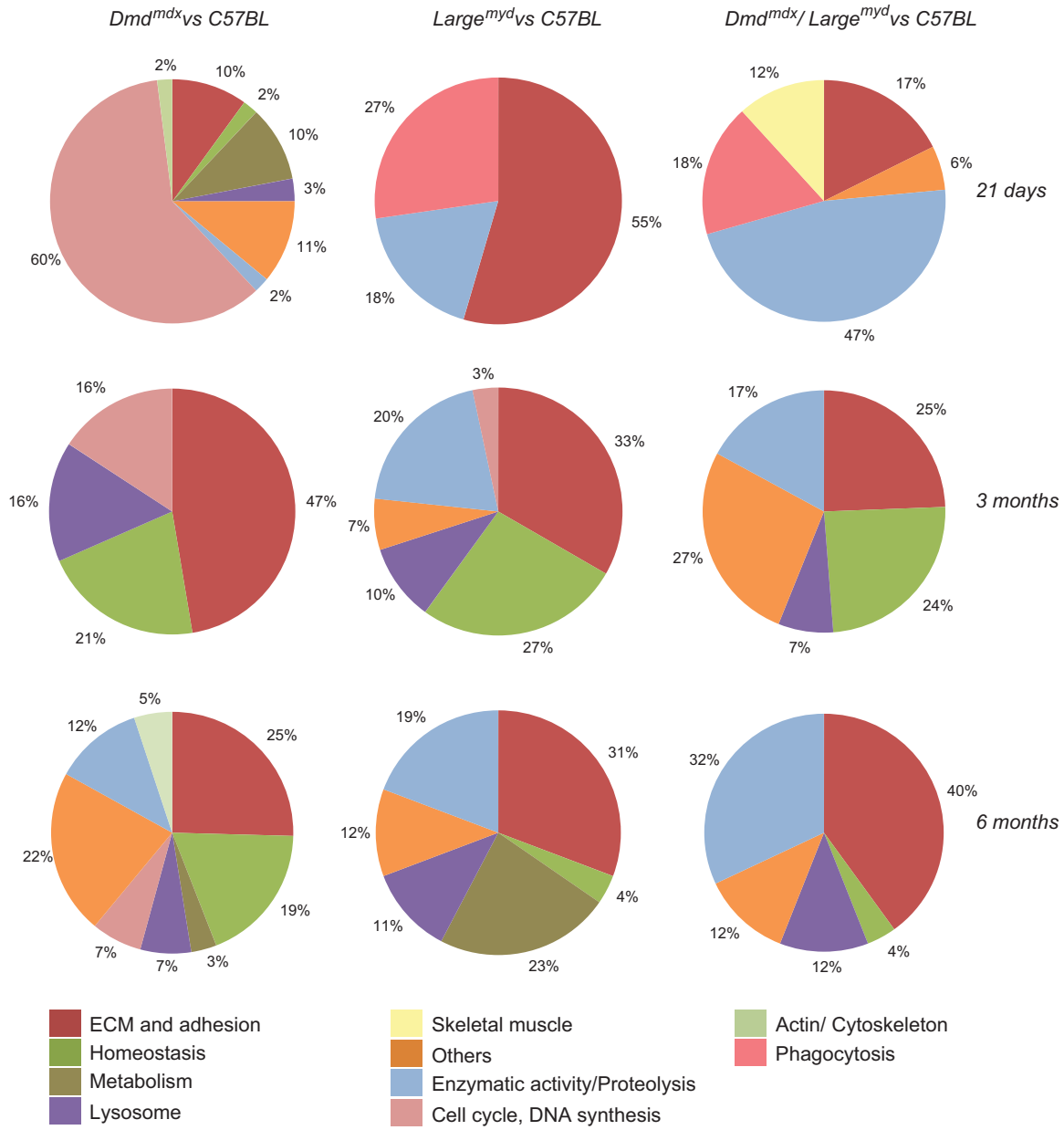

Figure 5 Enriched functional categories. Pie charts representing the enriched GO terms upregulated in all mice studied, excluding immune system and inflammation category.

Table 1 Number of differentially expressed genes in *Dmd*^{mdx}/*Large*$^{myd-/-}$ mice in relation to parental lineages

	21 Days		3 Months		6 Months	
Comparisons	*Up*	*Down*	*Up*	*Down*	*Up*	*Down*
Dmd^{mdx}/*Large*$^{myd-/-}$ × *Dmd*^{mdx}	6	124	38	15	11	25
Dmd^{mdx}/*Large*$^{myd-/-}$ × *Large*$^{myd-/-}$	8	8	9	3	32	1

Timp1, the inhibitor of MMP1, was already been seen upregulated on DMD patients, suggesting the reduction of *Mmp1* activity and consequently diminishing ECM removal and increasing fibrosis.[28] Elevated *Timp1* expression can include MMPs inhibition,[29,30] and two consequences directly related to the dystrophic process: intensify fibrosis and disturb regeneration, hampering cell migration by *Mmp9* inhibition.[28]

Myh3 converts ATP chemical energy into mechanic energy. It is a gene predominantly expressed in skeletal muscle and indispensable to muscle development and regeneration.[31] Therefore, its overexpression is a marker of regeneration, indicating the presence of immature muscle cells in the dystrophic muscles.[32]

Myl4, another regeneration marker overexpressed in the mouse models here studied, is related to myotube formation, as it is transcribed in skeletal muscle during the start of muscle differentiation.[33]

Tnnt2, coding for troponin type 2, is also expressed at the beginning of regeneration and was found overexpressed in DMD patients, which was associated with the active regeneration in dystrophic muscle.[34]

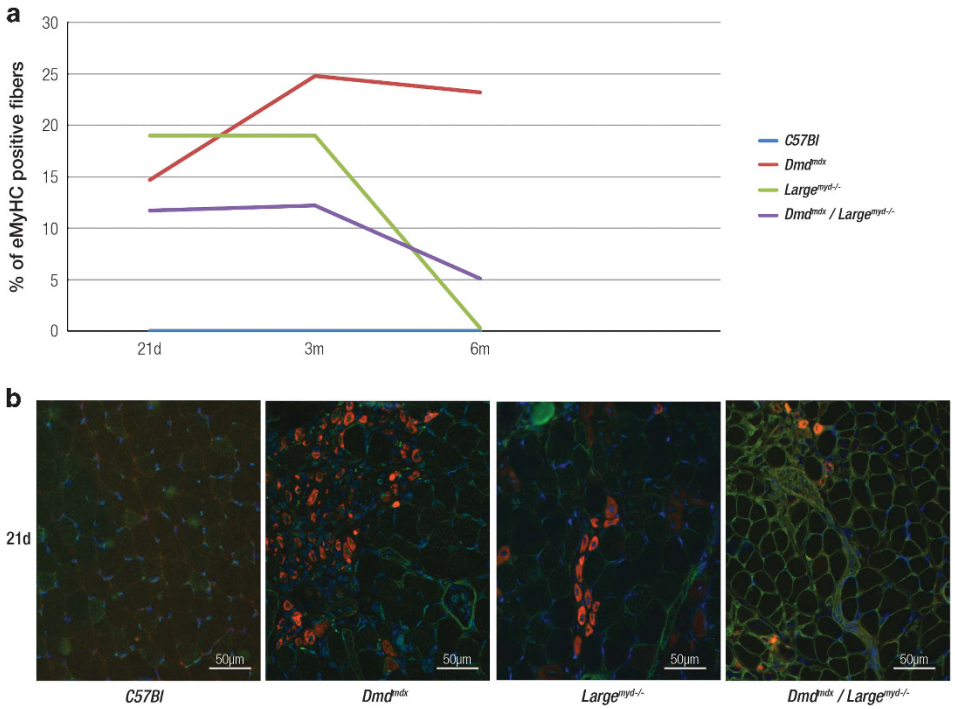

Figure 6 Proportion of positive eMyHC myofibers. (**a**) Percentage of positive fibers in the muscle of studied mice over time. (**b**) Representative images of eMyHC staining in muscles sections from 21-day-old animals.

Cathepsin S is a protease located in lysosome that cleaves antigenic proteins for MHC-II presentation. The altered expression of the *Ctss* gene was seen in Dmd^{mdx} transcriptome, and it was proposed that cathepsin can modulate elastin production and its deposition in the muscle. This could indicate an apparent response mechanism to reinforce the links between the ECM and the cytoskeleton, protecting the muscular fibers against contraction damages.[20]

A higher expression of *Gpnmb* (osteoactivin) in dystrophic mice[35] and DMD patients[36] was previously described. Osteoactivin is implicated in the differentiation and function of many cell types.[37] Although its expression is induced in the denervated muscle,[38] its superexpression protects the muscle from degeneration and fibrosis.[39]

In summary, the genes superexpressed in all the dystrophic processes are involved in the regeneration process and ECM remodeling. Among the remaining 23 DEGs, some do not have its function yet determined, and many of them are related to the immune system process.

Age effect

By analyzing the genes shared between the three dystrophic lineages, at each age, we found that the total of shared DEGs increases with aging. So, we checked which are the genes differentially expressed specifically at each individual age in the three lineages.

Seventeen DEGs are shared only by the 21-day-old animals; we highlight the following: *Ccna2*, *Ccnb1/Gm5593* and *Cdkn1A* that have functions in cell cycle, suggesting an increase in cell division. *Myh8* can represent the muscle immaturity or the presence of new fibers from regeneration. *Spp1* codifies the protein osteopontin secreted by myoblasts. Osteopontin stimulates myoblasts proliferation[40] and was described as an inflammatory player in dystrophic muscle.[16,41] Recently, it was reported that after a lesion, osteopontin expression is induced and promotes muscle inflammation, necrosis and regeneration, acting as an important factor to muscle remodeling.[40] The overexpression of *Spp1* can be related to the intense degeneration and subsequent regeneration present in the muscle at this age.

At 3 months of age, we highlight the gene *Capn6*, which is predominantly expressed in embryonic muscles and placenta. Calpain 6 has been considered as a differentiation and growth suppressor of skeletal muscle, since its deficiency can promote muscular differentiation in the development as well as in regeneration, modulating basal functions like cell division.[42] We found 55 DEGs for the age of 6 months. Among them are *Col3a1*, *Col6a3* and *Col8a1*, all overexpressed, reflecting fibrosis formation. *Mstn* is downregulated, which can be an attempt to help regeneration in adult animals.

Characterization of each dystrophic strain

Dmd^{mdx} lineage has the largest number of total deregulated genes (577, Figure 1a) and the majority is concentrated at 21 days old. In terms of gene transcription, the juvenile phase is characterized by an important disturbance of gene expression, followed by stabilization at adult life. Besides this tendency of a smaller number of DEGs with disease's progression being also reported in previous articles, the number of genes found in this work is superior.

$Large^{myd-/-}$ mice have a total of 343 DEGs (Figure 1a). The number of deregulated genes increases with aging. Unlike Dmd^{mdx}, who showed the higher number of DEGs at the age of 21 days, $Large^{myd-/-}$ presented the higher alteration in the transcriptome at 6 months of age, when $Large^{myd-/-}$ mice are more impaired.

$Dmd^{mdx}/Large^{myd}$ showed a similar total number of DEGs to $Large^{myd-/-}$ (376 genes, Figure 1). However, in $Large^{myd-/-}$ a gradual increase in the number of DEGs occurs with age, while in $Dmd^{mdx}/Large^{myd}$ an increase in the number of DEGs occurs after 21 days old, and remains stable at the adult phase (3 and 6 months).

The Gene Ontology (GO) classification showed that, for all lineages and ages, the most representative category was *Immune system and Inflammation*, accounting for 55–88% of all enriched GO themes.

The role of immune system in the pathogenesis of muscular dystrophies is well known; the marked representation of genes related to immunological and inflammatory functions was also reported in previous gene expression studies. The expression profiling of medial gastrocnemius of *Dmd*^{mdx} mice revealed that most induced genes belongs to immune response and inflammation.[20] Porter *et al*[16] found 242 DEGs in the *Dmd*^{mdx} gastrocnemius, of which 29.8% were classified as inflammatory genes, determining a chronic inflammatory response. Similar results were reported also by other authors.[18,19,43] Nevertheless, the proportion of immunity and inflammation genes we see in our data is higher to what has been published so far, reaching up 88% of the enriched functions

Observing the other categories apart (Figure 4), we could extract some interesting information about the main biological process affected beyond immune system and inflammation.

For 21-day-old *Dmd*^{mdx}, it is remarkable the great proportion of functions related to cell cycle and DNA synthesis. Although it accounts for about 60% of the functional categories in this model, this category is poorly represented in other lineages. It is known that at this specific age the muscle starts to present the first signs of the disease, distinguished by an intense degeneration.[4] Consequently, the regenerative process is activated, including the activation and proliferation of new cells, that here we see represented by the rise in the expression of cell-cycle genes. Thus, further studies of the genes implicated on these functions will help to understand the milder phenotype of this mouse, revealing possible therapeutic markers.

Comparing *Dmd*^{mdx}with *Large*^{myd} and *Dmd*^{mdx}/*Large*$^{myd-/-}$ strains at the same age of 21 days, the reduction in the participation of cell-cycle pathways in the more severely affected strains is accompanied by a proportional increase in the *ECM and adhesion* pathway in *Large*^{myd}, and of *Phagocytosis* and *Enzymatic activity/Proteolysis* categories in *Dmd*^{mdx}/*Large*$^{myd-/-}$. Considering that *Large* mutation affects the link of α-dystroglycan protein with the basal lamina protein laminin 2, it is expected that other ECM components would be more perturbed.

For the ages of 3 and 6 months, more homogenous profiles were observed, comparing the three lineages. In this sense, the largest differences along the ages occur in the *Dmd*^{mdx}model.

Interestingly, in spite of carrying the same mutation in the dystrophin gene, in the same C57BL background, *Dmd*^{mdx}/*Large*$^{myd-/-}$ did not present any cell division function enriched observed in the parental *Dmd*^{mdx}, indicating that the double-mutant lineage did not inherit from *Dmd*^{mdx} this outstanding feature. This possibly implies in a deficient activation of the regeneration mechanism in this model, which could explain the worst aspect of the tissue observed in histological studies.[9]

Another important category in the parental *Large*^{myd} lineage, *ECM and adhesion,* also seems not be inherited by the double-mutant strain. At the age of 21 days, this category corresponds to 55% in *Large*^{myd}, but only to 17% in the *Dmd*^{mdx}/*Large*$^{myd-/-}$.

The proportionally second most representative category for *Dmd*^{mdx}/*Large*$^{myd-/-}$ is *Enzymatic activity/proteolysis*. The genes involved in degradation of the ECM included *Mmp19* (+1.8), *Mmp12* (+1.8), *Mmp8* (+4.6) and *Timp1* (+2.7). Thus, the increased expression of these genes is a response to the fibrotic tissue deposition that is already occurring in the muscle. The remaining genes in this category are the genes *Wfdc17* (+2.4), *Serpine1* (+1.9), *Serpina3* (+4.2), *Serpina3k* (+1.9), *Lgmn* (+1.5), *Ctss* (+2.2), *Cdkn1A* (+1.6), *Stfa2/Stfa2l1* (+4.8), *R3hdml* (+1.6), *Asprv1* (+2.6), *Adam8* (+2.2) and *Dpep2* (+2.0) (these genes are listed in Supplementary Table S7). The gene *Stfa2/Stfa2l1* encodes an endopeptidase inhibitor and was the gene with the great fold change in the *Dmd*^{mdx}/*Large*$^{myd-/-}$ with 21 days of age (+4.8); *R3hdml* is also an inhibitor of peptidase activity, and the peptidase ASPRV1. To our knowledge, there is no many information about the functions of these genes in previous studies.

Dmd^{mdx}/Large$^{myd-/-}$ versus parental strains

When compared with parental strains, *Dmd*^{mdx}/*Large*$^{myd-/-}$ showed the largest differences in relation to 21-day-old *Dmd*^{mdx}, with 130 DEGs, mainly downregulated (124 genes). Considering that at this age 481 DEGs are upregulated in the *Dmd*^{mdx} versus control, it would be expected that these 124 downregulated genes in *Dmd*^{mdx}/*Large*$^{myd-/-}$ would be among them. However, only 41 genes (from 124) are upregulated in the *Dmd*^{mdx} vs control and downregulated in the *Dmd*^{mdx}/*Large*$^{myd-/-}$ vs *Dmd*^{mdx}. Therefore, in the double mutant, these genes have a distinct behavior from the parental lineage. It would also be expected that part of these deregulated genes would be involved with cell proliferation functions, which have a strong participation in *Dmd*^{mdx}. However, the majority of these genes execute functions related to immunological pathways. Even though, four genes are involved in the positive regulation of cell proliferation: *Clec11a*, *CD28*, *Derl2* and *Il15*; and two to the cell cycle: *Haus8* and *Gadd45gip1*. Among seven other genes including *Mup1*, *Ifi204*, *Retnla*, *Slamf7*, *Cyp4f2*, *Fcrls* and *Oas,* only one presented a muscle correlated function. This gene, *Ifi204,* produces a protein at higher levels during myoblasts fusion, under the action of MyoD. *Ifi204* overexpression accelerates myoblasts fusion,[44] as it reduces Id proteins levels, known for inhibit muscle differentiation by blocking MyoD and other myogenic proteins.[45] On the basis of this information, we hypothesize that *Ifi204* downregulation in *Dmd*^{mdx}/*Large*$^{myd-/-}$ could hamper myogenesis, unlike *Dmd*^{mdx} that upregulates *Ifi204* and shows a better regenerative potential.

The overlap of the three gene lists of *Dmd*^{mdx}/*Large*$^{myd-/-}$ showed that 59 genes are deregulated independently of age (Figure 2a). Thirty-one of these genes are those present in all the dystrophic lineages. From the remaining 28, seven are also deregulated in *Large*$^{myd-/-}$ and 20 in *Dmd*^{mdx}, so just 1 gene is always upregulated only in *Dmd*^{mdx}/*Large*$^{myd-/-}$. Thus, the combination of two mutations does not cause much more alterations in the transcriptome than what happen in the parental lineages. Nevertheless, a sum of the altered genes in each lineage does not occur, because the number of DEGs in *Dmd*^{mdx}/*Large*$^{myd-/-}$ is inferior to the sum of DEGs from *Dmd*^{mdx}and *Large*$^{myd-/-}$. Thus, only part of the genes with differential expression in the parental will also be altered in double-mutant mice. In spite of that, comparing enriched KEGG pathways (data not shown) for *Dmd*^{mdx}/*Large*$^{myd-/-}$ and *Large*$^{myd-/-}$, both lists are very similar and pathways activated in *Dmd*^{mdx} are not activated in *Dmd*^{mdx}/*Large*$^{myd-/-}$. Consequently, we conclude that *Dmd*^{mdx}/*Large*$^{myd-/-}$ expression profile is more similar to *Large*$^{myd-/-}$.

The unique DEG in the *Dmd*^{mdx}/*Large*$^{myd-/-}$ strain in all time points is *Lilrb3*, which encodes a receptor that binds MHC class I molecules in immune cells, transducing a negative signal to inhibit the stimulation of an immune response. It is believed that this receptor controls inflammatory responses and cytoxicity, limiting auto reactivity.[46] There is no information in the literature that helps to correlate *Lilrb3* upregulation with the double-mutant phenotype.

Muscle regeneration

At overall, *Dmd*^{mdx} mice presented a higher proportion of regenerating fibers than the other two lineages, increasing with the age, while in *Large*^{myd} and *Dmd*^{mdx}/*Large*^{myd} mice the percentage of positive fibers was smaller, and reduced drastically with aging (Figures 6a and b). The higher quantity of regenerating fiber corroborates the transcription data, confirming that *Dmd*^{mdx} has better tools to deal with muscle degeneration and that this was not inherited by *Dmd*^{mdx}/*Large*^{myd}. Despite the higher expression of genes related to cell cycle is seen at

21-day-old *Dmd*^{mdx}, the higher proportion of regenerating fibers is observed on older ages. This indicates that the transcription of these genes at early stages is a preparation to the formation of new fibers in later stages of the disease, resulting in a better phenotype, which is seen neither in *Large*^{myd} nor in *Dmd*^{mdx}/*Large*^{myd}.

In conclusion, this is the first report about the global gene expression pattern of mice models for congenital muscular dystrophy (*Large*^{myd}) and the double-mutant *Dmd*^{mdx}/*Large*^{myd}. By comparing the three studied profiles, we showed that the alterations in the biological processes due to the dystrophic process are very similar, the double mutant presents few differences in relation to its parental strains, mainly the *Dmd*^{mdx}; and it is more similar to *Large*^{myd}. The intense regeneration in the *Dmd*^{mdx} model involves a large number of activated genes, which were not differentially expressed in the other two strains.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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