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Life or death by NFκB, Losartan promotes survival in *dy²ᴶ/dy²ᴶ* mouse of MDC1A

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Inflammation and fibrosis are well-defined mechanisms involved in the pathogenesis of the incurable Laminin α2-deficient congenital muscular dystrophy (MDC1A), while apoptosis mechanism is barely discussed. Our previous study showed treatment with Losartan, an angiotensin II type I receptor antagonist, improved muscle strength and reduced fibrosis through transforming growth factor beta (TGF-β) and mitogen-activated protein kinases (MAPK) signaling inhibition in the *dy²ᴶ/dy²ᴶ* mouse model of MDC1A. Here we show for the first time that Losartan treatment up-regulates and shifts the nuclear factor kappa B (NFκB) signaling pathway to favor survival *versus* apoptosis/damage in this animal model. Losartan treatment was associated with significantly increased serum tumor necrosis factor alpha (TNF-α) level, p65 nuclei accumulation, and decreased muscle IκB-β protein level, indicating NFκB activation. Moreover, NFκB anti-apoptotic target genes TNF receptor-associated factor 1 (TRAF1), TNF receptor-associated factor 2 (TRAF2), cellular inhibitor of apoptosis (cIAP2), and Ferritin heavy chain (FTH1) were increased following Losartan treatment. Losartan induced protein expression toward a pro-survival profile as BCL-2 expression levels were increased and Caspase-3 expression levels were decreased. Muscle apoptosis reduction was further confirmed using terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assay. Thus, along with TGF-β and MAPK signaling, NFκB serves as an important regulatory pathway which following Losartan treatment promotes survival in the *dy²ᴶ/dy²ᴶ* mouse model of MDC1A.
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Congenital muscular dystrophy type 1A (MDC1A) is one of the most common forms of congenital muscular dystrophies (CMDs). Clinical symptoms are severe hypotonia, muscle weakness, and delayed motor milestones.[1] Typically, the children do not achieve independent ambulation and respiratory failure is followed by death in the second or third decade of life.[2,3] MDC1A is caused by mutations in the *LAMA2* gene, encoding the heavy chain of laminin-2.[4] Muscle biopsies are characterized by muscle fiber necrosis, inflammation, apoptosis, and fibrosis.[1–3,5] Despite extensive advances in its diagnosis, MDC1A remains an incurable disease.[2,6]

The *dy²ᴶ/dy²ᴶ* mouse is a useful model to study the pathophysiology of MDC1A and the effect of various therapeutic agents.[7–9] This mouse has a mutation in the *LAMA2* gene resulting in abnormal splicing of the laminin-α2 polypeptide and a moderate to severe phenotype characterized by development of muscle weakness at about 3 weeks of age, which progressively worsens.[7,10] The pathology of *dy²ᴶ/dy²ᴶ* skeletal muscle is quite similar to children with MDC1A, showing muscle fiber degeneration, necrosis, and apoptosis, followed by inflammation and fibrosis.[7,10–12]

Previous studies among ours have shown that the pathogenesis of muscular dystrophies involves coordinated activation of multiple key signaling pathways.[9,13–16] Nuclear factor kappa B (NFκB) has been described as a significant

transcription factor that regulates the expression of muscle proinflammatory cytokines.[16]

Early studies have shown elevated NFκB levels in skeletal muscle of *mdx* mice, the mouse model for Duchenne muscular dystrophy (DMD), and in inflammatory myopathies.[17–21] NFκB activation is thought to contribute to the deterioration of skeletal muscle pathology and muscle loss in DMD.[18] However, NFκB seems to have a multifaceted regulatory role and may show protective activity in different disorders. Baghdiguian *et al.*[22,23] have shown that a certain level of NFκB activity is required to protect myofibers from apoptosis in a Calpain-3 mouse model of Limb girdle muscular dystrophies (LGMDs). Several studies indicate that NFκB activation has a positive role in cell survival by inducing transcription of several survival genes.[24,25] However, there is only limited data regarding NFκB's role in MDC1A.

Losartan, an angiotensin II type 1 receptor antagonist, is a commercially available and extensively used medication for hypertension with a low side effect profile, occasionally used in childhood.[26] In our previous study, we showed that Losartan treatment was associated with significant impressive improvement in muscle strength and amelioration of fibrosis in the *dy²ᴶ/dy²ᴶ* mouse model of MDC1A, through inhibition of transforming growth factor beta (TGF-β) and the mitogen-activated protein kinases (MAPK) signaling pathway.[9]

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Here, we demonstrate NFκB signaling pathway involvement in the pathophysiology of the dy^{2J}/dy^{2J} mouse model, mediating decreased apoptosis and promoting muscle cell survival following Losartan treatment. Reduced apoptosis and pro-survival NFκB target genes activation following treatment suggest a key regulatory role for the NFκB signaling pathway in this disorder.

Results

Losartan treatment modifies TNF-*α* expression. The tumor necrosis factor alpha (TNF-*α*) serum level was significantly increased in treated dy^{2J}/dy^{2J} mice compared with untreated dy^{2J}/dy^{2J} mice (5.1 ± 0.96 pg/ml *versus* 2.05 ± 0.58 pg/ml; *$P < 0.05$). TNF-*α* serum level was also significantly increased in Losartan-treated WT mice (treated: 5.69 ± 0.49 pg/ml *versus* untreated: 2.42 ± 0.56 pg/ml; **$P < 0.0005$). These results are presented in Figure 1a. As these results were unexpected, we further examined the effect of Losartan on TNF-*α* transcript levels, using quantitative real-time PCR (TaqMan). The *TNF-α* mRNA level was unchanged in untreated dy^{2J}/dy^{2J} mice compared with WT groups, but was significantly increased in treated dy^{2J}/dy^{2J} mice hind limb muscles (*$P < 0.05$; Figure 1b). Since TNF-*α* induces NFκB target gene expression, we analyzed NFκB activity and its downstream effects following Losartan treatment in more detail.

Losartan treatment altered NFκB activation. Using western blot analysis we found significantly decreased expression of the classic NFκB inhibitor, IκB-*α* protein, in hind limb muscles of both treated and untreated dy^{2J}/dy^{2J} mice compared with WT mice (untreated dy^{2J}/dy^{2J}: 0.74 ± 0.07-fold *versus* untreated WT: 1 ± 0.08-fold; $P < 0.005$; and treated dy^{2J}/dy^{2J}: 0.72 ± 0.05-fold *versus* treated WT: 0.99 ± 0.13-fold; $P < 0.005$). However IκB-*α* protein expression was unchanged in dy^{2J}/dy^{2J} mice following Losartan treatment, indicating pre-existing NFκB activation in untreated dy^{2J}/dy^{2J} mice (Figure 2a).

We next measured protein expression of IκB-*β*, an additional classic NFκB inhibitor. Only following Losartan treatment was IκB-*β* expression significantly decreased in both dy^{2J}/dy^{2J} and WT mice (treated dy^{2J}/dy^{2J}: 0.32 ± 0.05-fold *versus* untreated dy^{2J}/dy^{2J}: 0.53 ± 0.07-fold; *$P < 0.05$; treated WT: 0.37 ± 0.04-fold *versus* untreated WT: 1 ± 0.15-fold; **$P < 0.005$) (Figure 2b). Thus in untreated dy^{2J}/dy^2 NFκB activation follows decreased expression of IκB-*α*, however after Losartan treatment NFκB activation follows decreased expression of both inhibitors; IκB-*α* and IκB-*β*.

In the next step, immunofluorescence staining of quadriceps muscles revealed significantly higher accumulation and co-localization of NFkB p65 subunit in the muscle nucleus of treated and untreated dy^{2J}/dy^{2J} mice, compared with WT mice (untreated dy^{2J}/dy^{2J}: $20.57 \pm 1.25\%$ *versus* WT: $0.166 \pm 0.166\%$; $P < 0.00001$ and treated dy^{2J}/dy^{2J}: $20.71 \pm 2.59\%$ *versus* WT: $0.66 \pm 0.49\%$; $P < 0.00001$) (Figure 2c). These findings confirm NFκB activation in dy^{2J}/dy^{2J} mice with and without treatment.
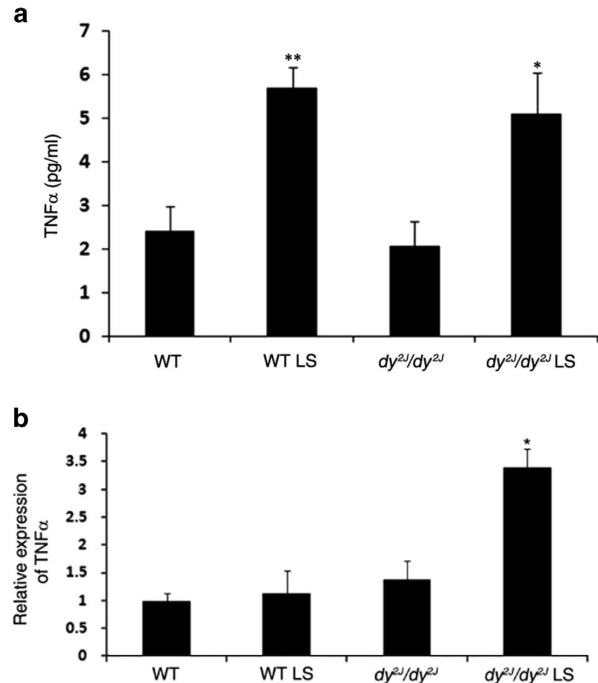
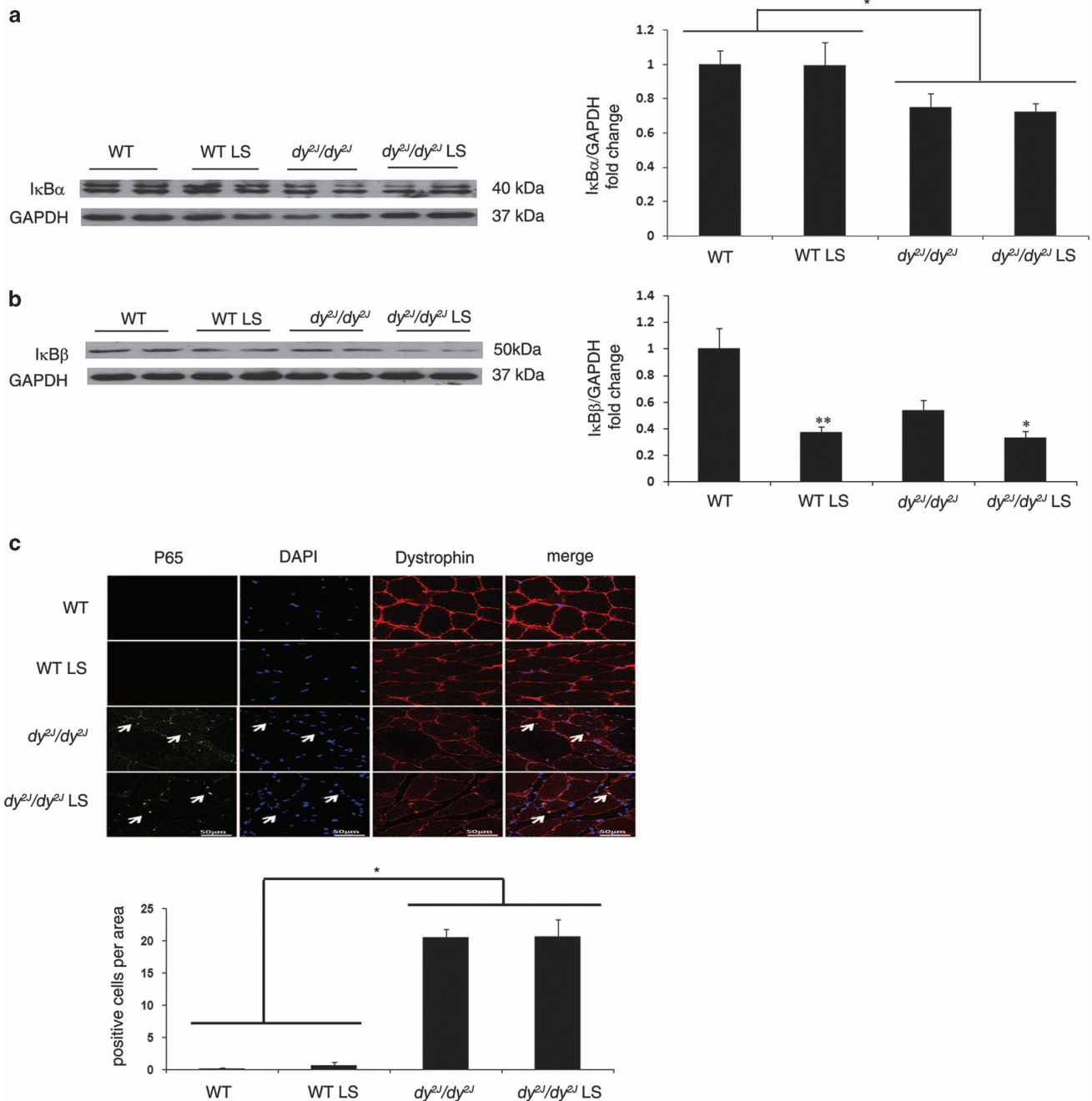**Figure 1** TNF-*α* activity in dy^{2J}/dy^{2J} and WT mice following Losartan treatment. (**a**) Losartan significantly increased serum TNF-*α* level in treated compared with untreated dy^{2J}/dy^{2J} mice (*$P < 0.05$). Losartan also increased TNF-*α* levels in treated compared with untreated WT mice (**$P < 0.0005$). Each bar represents the mean $\pm$ S.E.M. of nine mice at 19 weeks of age. (**b**) Total RNA was extracted from Hind limb muscles of WT and dy^{2J}/dy^{2J} mice. Quantitative real-time PCR (TaqMan) of *TNF-α* mRNA expression levels was determined. Significant increased mRNA level of *TNF-α* was noted upon Losartan treatment in dy^{2J}/dy^{2J} mice. Expression levels were normalized to the housekeeping gene, TATA box binding protein (*TBP*) mRNA level (*$P < 0.05$). Results represent the mean $\pm$ S.E.M. of five mice

Losartan treatment upregulates several pro-survival NFκB target genes. Since NFκB appears to be involved in the regulation of both apoptosis and cell survival, we examined the effect of Losartan on NFkB target genes using quantitative real-time PCR (TaqMan). Anti-apoptotic NFkB target genes TNF receptor-associated factor 1 and TNF receptor-associated factor 2 (*TRAF1* and *TRAF2*), cellular inhibitor of apoptosis (*cIAP2*), and Ferritin heavy chain (*FTH1*) were analyzed. We found that mRNA expression of *TRAF1*, an adaptor protein required for optimal anti-apoptotic NFκB activation, was significantly increased in treated WT and in both treated and untreated dy^{2J}/dy^{2J} compared with the untreated WT mice (*$P < 0.01$,**$P < 0.05$; Figure 3a). Because *TRAF1* recruits *TRAF2* and cIAPs to activate the anti-apoptotic process, we next measured the transcript levels of *TRAF2* and *cIAP2* genes. *TRAF2* and *cIAP2* genes were significantly increased in hind limb muscles of both dy^{2J}/dy^{2J} and WT mice following treatment (*TRAF2*; *$P < 0.005$, **$P < 0.05$ and *cIAP2*; *$P < 0.01$, **$P < 0.05$) (Figures 3b and c). *FTH1* gene expression was significantly increased following treatment in dy^{2J}/dy^{2J} mice (*$P < 0.01$), with no significant increase in the WT mice (Figure 3d). All of these findings suggest that Losartan upregulates NFκB pro-survival target genes.

Losartan treatment increases anti-apoptotic protein BCL-2 expression and decreases the pro-apoptotic protein Caspase-3 expression. Next using western blot analysis we examined the hind limb expression of B-cell lymphoma 2 (BCL-2), an anti-apoptotic protein. BCL-2 expression was significantly higher in Losartan treated

Figure 2 NFκB signaling pathway activity in dy^{2J}/dy^{2J} and WT mice. (**a**) Representative western blot gel and densitometry graph of NF-kappa-B inhibitor alpha (IkB-α) expression in WT and dy^{2J}/dy^{2J} mice. Significant reduction in IkB-α was noted in untreated and treated dy^{2J}/dy^{2J} mice compared with WT groups (*$P < 0.005$). (**b**) Representative western blot gel and densitometry graph of NF-kappa-B inhibitor beta (IkB-β) expression in WT and dy^{2J}/dy^{2J} mice. Significant reduction in IkB-β was noted in treated compared with untreated dy^{2J}/dy^{2J} mice (*$P < 0.05$). Losartan treatment was also associated with decreased IkB-β in treated WT mice compared with the untreated group (**$P < 0.005$). Results of IkB-α and IkB-β levels were obtained from densitometric analysis and expressed as ratio of IkB-α-β/GAPDH and as change fold over control (WT group). These results represent three independent experiments. Each bar represents the mean $\pm$ S.E.M. of 12 mice for IkB-α and 11 mice for IkB-β. (**c**) Intracellular localization of p65 using Immunofluorescence analysis. Expression of p65 was analyzed using anti-Alexa-647 (yellow fluorescence) antibody. Dystrophin staining as a skeletal muscle marker was analyzed using anti-cy2 (red fluorescence) antibody, and DAPI staining (blue fluorescence) was used as nuclear staining. When p65 protein is localized to the muscle nucleus, Alexa-647, cy2, and DAPI are merged. The quadriceps muscle of untreated and treated dy^{2J}/dy^{2J} mice showed nuclear localization of p65 demonstrating NFkB activation. Lack of p65 in the nucleus was illustrated in untreated and treated WT groups. Scale bar, 50 μm. Each bar represents the mean $\pm$ S.E.M. of five fields per mice of six mice in the WT groups and seven mice in the dy^{2J}/dy^{2J} groups (*$P < 0.00001$)
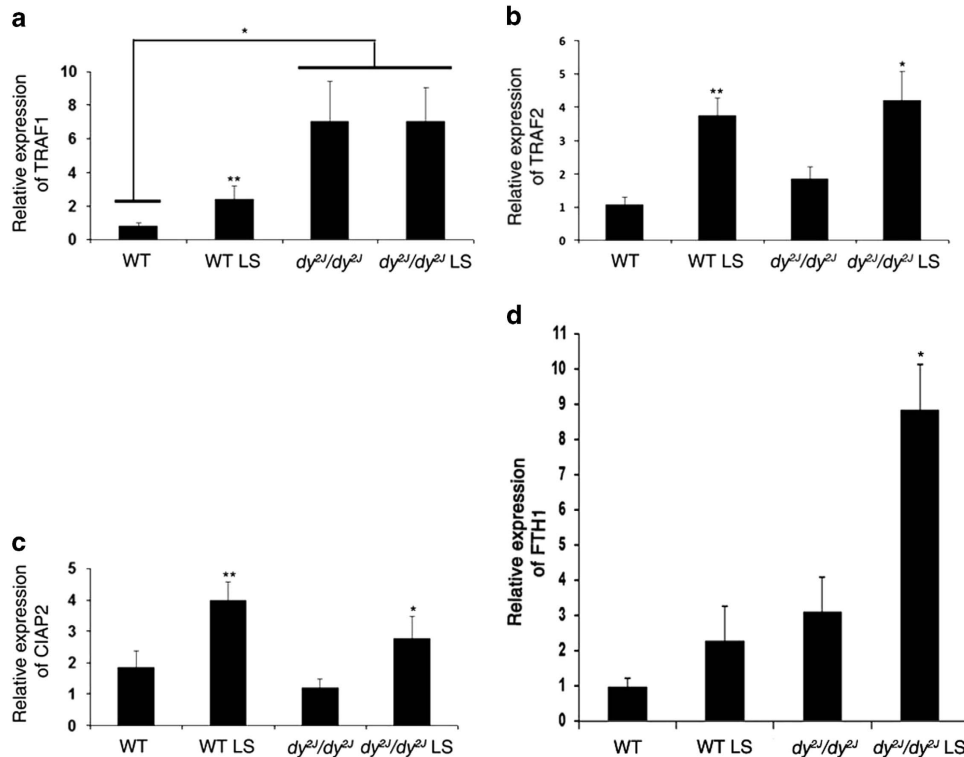
Figure 3 Losartan modified pro-survival/anti-apoptotic NFκB signaling target genes. Total RNA was extracted from Hind limb muscles of WT and dy^{2J}/dy^{2J} mice. Quantitative real-time PCR (TaqMan) of (**a**) *TRAF1*, (**b**) *TRAF2*, (**c**) *cIAP2*, and (**d**) *FTH1* mRNA expression levels were determined. A significantly increased mRNA level of the anti-apoptotic gene *TRAF1* was noted in treated WT and in both treated and untreated dy^{2J}/dy^{2J} compared with untreated WT mice (*$P < 0.01$,** $P < 0.05$). Significantly increased mRNA levels of the anti-apoptotic genes, *TRAF2* and *cIAP2*, were noted in Losartan treated WT and dy^{2J}/dy^{2J} mice (*TRAF2*; *$P < 0.005$, **$P < 0.05$ and *cIAP2*; *$P < 0.01$, **$P < 0.05$). Significantly increased mRNA levels of the anti-apoptotic genes *FTH1* were noted in Losartan-treated dy^{2J}/dy^{2J} mice (*$P < 0.01$). Expression levels were normalized to the housekeeping gene, TATA box binding protein (*TBP*) mRNA level. Results represent the mean ± S.E.M. of five mice for *TRAF1*, *TRAF2*, *cIAP2*, and *FTH1*

compared with untreated dy^{2J}/dy^{2J} mice (treated dy^{2J}/dy^{2J}: 0.90 ± 0.052-fold *versus* untreated dy^{2J}/dy^{2J}: 0.59 ± 0.071-fold; *$P < 0.01$). There was no significant difference in BCL-2 expression between treated and untreated WT mice (Figure 4a).

As for the protein expression level of the pro-apoptotic protein Caspase-3, Losartan treatment reduced significantly its expression in treated compared with untreated dy^{2J}/dy^{2J} mice (treated dy^{2J}/dy^{2J}: 3.9 ± 1.3-fold *versus* untreated dy^{2J}/dy^{2J}: 10 ± 1.9-fold; *$P < 0.0001$) and in treated compared with untreated WT mice (treated WT: 0.3 ± 0.1-fold *versus* untreated WT: 1 ± 0.02-fold; **$P < 0.0001$ Figure 4b). Taken together, these results suggest that Losartan treatment modifies NFκB signaling toward pro-survival/anti-apoptotic pathway.

Losartan reduces TUNEL-positive muscle cells. In order to confirm NFκB involvement in apoptosis signaling, we used *in situ* DNA nick-end labeling (TUNEL), DNA fragmentation assay TUNEL analysis (Figure 5) showed significant reduction of TUNEL-positive cells in quadriceps muscles of Losartan treated compared with untreated dy^{2J}/dy^{2J} mice, indicating apoptosis (treated dy^{2J}/dy^{2J}: $2.6 \pm 0.35\%$ *versus* untreated dy^{2J}/dy^{2J}: $10.14 \pm 1\%$; *$P < 0.0001$). Almost no TUNEL-positive cells were found in untreated and treated WT groups.

Discussion

CMDs are genetically heterogeneous diseases, which result in severe disability and premature death. Muscle fibrotic tissue accumulation and progressive skeletal muscle strength reduction characterize both children and the dy^{2J}/dy^{2J} mouse model of MDC1A, one of the most frequent forms of CMD. We previously showed that Losartan treatment significantly increased both fore and hind limb muscle strength, with reduced collagen accumulation and fibrotic markers, in dy^{2J}/dy^{2J} mice skeletal muscle. This clinical and histological improvement was associated with TGF-β and MAPK signaling pathway inhibition, as Losartan was associated with reduced expression of the regulatory Smad; P-Smad2 and 3 and increased expression of the inhibitory Smad; Smad7. Furthermore, Losartan was associated with significant reduction of the three parallel MAPK signaling pathways P-ERK1/2, P-JNK, and P-p38.[9]

Our current study sheds new light on the NFκB signaling pathway and its involvement in the pathophysiology of the dy^{2J}/dy^{2J} mouse model of MDC1A, mainly through a new insight into the apoptosis pathway. In addition, these data reveal a new role for Losartan with regard to NFκB signaling and apoptosis in this disorder.

In this study, while evaluating Losartan's effect on cytokine levels in mice serum, we found an unexpected significant

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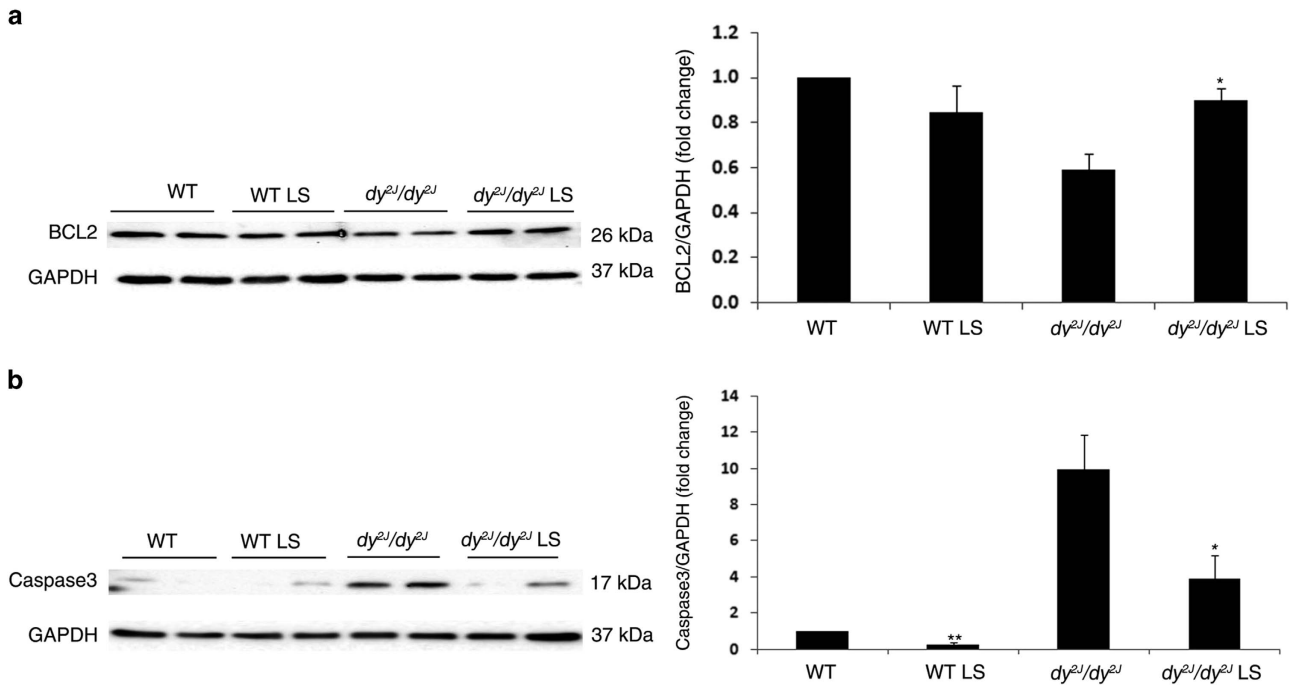
Figure 4 Increased protein expression of anti-apoptotic BCL-2 and decreased protein expression of pro-apoptotic Caspase 3 following Losartan treatment. (**a**) Representative western blot gel and densitometry graph of BCL-2 expression in WT and dy^{2J}/dy^{2J} mice. A significantly higher BCL-2 protein expression level was noted in treated compared with untreated dy^{2J}/dy^{2J} mice (*$P < 0.01$). (**b**) Representative western blot gel and densitometry graph of Caspase-3 expression in WT and dy^{2J}/dy^{2J} mice. A significantly lower Caspase-3 protein expression level was noted in treated compared with untreated dy^{2J}/dy^{2J} mice (*$P < 0.0001$). Results of BCL-2 and Caspase-3 levels were obtained from densitometric analysis and expressed as ratio of BCL-2 and Caspase-3 to GAPDH and as change fold over control (WT group). These results represent three independent experiments. Each bar represents the mean ± S.E.M. of 12 mice for BCL-2 (*$P < 0.01$) and 12 mice for Caspase-3 (*/**$P < 0.0001$)
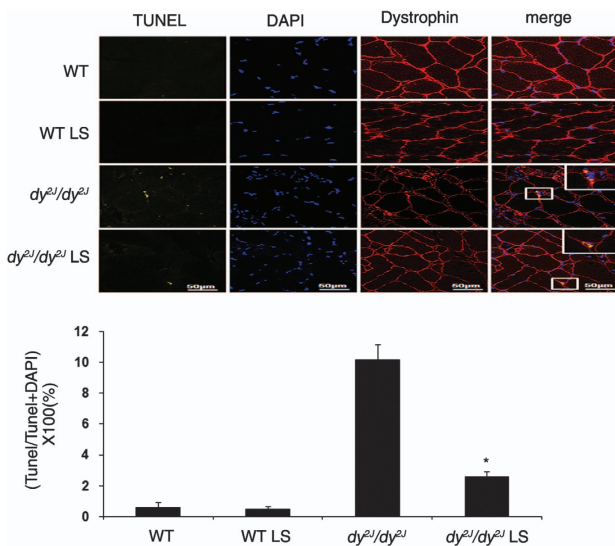
Figure 5 Losartan decreased apoptosis in dy^{2J}/dy^{2J} mice muscle. Expression of apoptosis was analyzed using TUNEL assay with an *In Situ* Cell Death Detection Kit. TUNEL-positive cells were stained in yellow. Dystrophin, as muscle marker, was analyzed using anti-Alexa-647 antibody (red), and DAPI staining (blue) was used as nuclear staining. The quadriceps muscle of Losartan-treated dy^{2J}/dy^{2J} mice showed significant reduction in TUNEL-positive cells compare with the untreated mice. Almost no TUNEL-positive cells were illustrated in untreated and treated WT groups. Scale bar, 50 μm. Each bar represents the mean ± S.E.M. of five fields per mice of six mice in the WT groups and seven mice in the dy^{2J}/dy^{2J} groups (*$P < 0.0001$)

increase in the TNF-α level following treatment in both WT and dy^{2J}/dy^{2J} mice (Figure 1a). TNF-α is known to mediate a variety of cellular responses including inflammation, necrosis, fibrosis, and apoptosis.[27] One major role of TNF-α is stimulation of the NFκB signaling pathway.[28,29] We therefore further investigated the NFκB signaling pathway involvement in MDC1A pathology.

NFκB is a transcription factor that in its resting state binds to inhibitory IκB proteins, keeping it inactive and localized to the cytoplasm. Following stimulation NFκB detaches from its inhibitors. The resulting free NFκB translocates into the nucleus where it activates or represses target genes.[17,30] Here we demonstrated NFκB activation in both treated and untreated dy^{2J}/dy^{2J} mice through IκB-α protein reduction and p65 (NFκB subunit) transcription factor accumulation in the nucleus of skeletal muscle cells (Figures 2a and c). Losartan-treated mice showed NFκB activity via reduction of an additional inhibitor, IκB-β (Figure 2b). These results may indicate different branches in the NFκB pathway are activated upon Losartan treatment compared with untreated dy^{2J}/dy^{2J} mice.

Previous studies have addressed Losartan's role in inhibition of NFκB inflammatory processes. They showed Losartan reduces and inhibits NFκB activity in muscle cells from porcine coronary artery,[31] and suppresses inflammation in aged rat kidney.[32] However, NFκB signaling regulates transcriptional

programs that are essential for the development and maintenance of the skeletal system,[33] epithelium,[34] and immune system,[35] which in turn impacts differentiation, proliferation, cell death, and survival.[36,37] Therefore, NFκB signaling pathways have a multifaceted regulatory role that can either mediate apoptosis or anti-apoptotic routes. NFκB signaling can also engage the Caspase signaling pathway to mediate cell apoptosis.[38,39] On the other hand, it can activate TNF receptor-associated factors (TRAFs) and cellular inhibitor of apoptosis (CIAPs) to suppress cell death.[21,24,25,40]

In Duchenne muscular dystrophy, NFκB activation is perceived as contributing to the deterioration of skeletal muscle pathology and skeletal muscle loss.[18] For example, deletion of a single allele of NFκB (RelA/p65 subunit) was sufficient to considerably reduce infiltration of macrophages, fiber necrosis and calcification in dystrophic muscle in mdx mice (DMD mouse model). In addition, NFκB inhibition augmented the regeneration of mdx mice myofibers.[41,42] Furthermore, overexpression in skeletal muscle of A20 protein, a potent negative regulator of NFκB, reduced chronic inflammation and muscle degeneration in mdx mice.[43,44]

However, in the current study, NFκB activity following Losartan treatment maintained survival and anti-apoptotic effects in dy^{2J}/dy^{2J} mice. Losartan treatment was associated with increased mRNA expression of pro-survival genes TRAF1, TRAF2, CIAP2, and FTH-1 (Figures 3a and d). These findings support other studies findings showing gene expression of TRAFs and CIAPs following NFκB activation, lead to inhibition of TNF-α induced cell death.[29,36,45,46] Cells lacking c-IAPs through genetic ablation or treated with IAPs antagonists have been shown to be more sensitive to TNF-α induced cell death through decreased NFκB survival mechanism.[25] Losartan's role in apoptosis inhibition was further demonstrated by a significant increase of anti-apoptotic BCL-2 protein expression in skeletal muscle of treated dy^{2J}/dy^{2J} mice (Figure 4a). BCL-2 has been shown to have an important role in increasing lifespan, growth rate, and reducing apoptosis following muscle-specific overexpression in Lama2 null mice.[47,48] Furthermore, overexpression of BCL-2 was found to ameliorate muscle weakness and reduce apoptosis in oculopharyngeal muscular dystrophy (OPMD) mouse model.[49] As for DMD, transgenic overexpression of BCL-2 did not improve muscle pathology in mdx mice,[48,50] and indeed the effect of Losartan in mdx mice is less pronounced than in dy^{2J}/dy^{2J} mice.[15]

We further investigated the anti-apoptotic effect of Losartan. Losartan treatment significantly decreased the pro-apoptotic protein Caspase-3 in dy^{2J}/dy^{2J} and WT mice (Figure 4b), and overall apoptosis was significantly decreased in Losartan-treated dy^{2J}/dy^{2J} mice as demonstrated by TUNEL-positive skeletal muscle cell reduction (Figure 5).

All of the above data together propose an important role for NFκB signaling in the pathophysiology of MDC1A, mainly in terms of its contribution to the apoptosis process. On the basis of current findings, we suggest that Losartan treatment shifts NFκB signaling to favor the survival route versus inflammation, fibrosis and apoptosis/damage in the dy^{2J}/dy^{2J} mouse model of MDC1A.

We therefore suggest a model regarding NFκB signaling activity following Losartan treatment. Losartan treatment results in increasing TNF-α, which in turn activates NFκB through IκB-α and IκB-β degradation, p65 nuclear accumulation and upregulation of pro-survival genes and proteins to mediate the anti-apoptotic effect of NFκB in the dy^{2J}/dy^{2J} mouse model of MDC1A (Figure 6).

In muscular dystrophies there are indications that apoptosis, beside necrosis, may contribute to muscle loss and dysfunction. In human and mouse models of muscular dystrophy signs of muscle cell death by apoptosis have been documented but not discussed in detail.[16,21,51,52] In this study, we show for the first time that apoptosis has an important role in dy^{2J}/dy^{2J} muscle pathology. Therefore, it seems that therapies designed to include apoptosis inhibition might be beneficial for patients with congenital muscular dystrophy. These new findings support our previous data that demonstrated significant improvement in animal muscle strength and reduced fibrosis following 12 weeks of Losartan treatment. A future more prolonged Losartan study will provide additional information regarding long-term benefit and survival in dy^{2J}/dy^{2J} mice. This trial provides further support for a Losartan therapeutic trial in children with MDC1A.

Materials and Methods

Mice population and treatment. Muscle tissues for this study were obtained from the mice used in a previous Losartan study[9] as follows; C57BL/6J Lama2dy-2J heterozygote mice (Jackson Laboratories, Bar Harbor, ME, USA) were bred at the Hebrew University specific pathogen-free animal housing facility. The joint ethics committee of Hebrew University and Hadassah Medical Center (accredited by AAALAC) approved the study protocol for animal welfare (permit number: 122.03-04). Mice were maintained under standard conditions, 23 ± 1 °C, 12- h light cycle (0700–1900 h), with ad libitum access to food and drink. Delineation between the Lama2dy-2 J (dy^{2J}/dy^{2J}) affected mice, heterozygous for the LAMA2 gene mutation, and wild-type C57BL/6 J (WT) mice was detected by PCR.[53] WT and dy^{2J}/dy^{2J} mice received 0.6 g/l Losartan (Merck Sharp & Dohme, West Point, PA, USA) in their drinking water or placebo as a control, The mice were treated for 12 weeks from 6 weeks of age ($n = 12$/group; each group consisted of 6 male and 6 female mice).

Cytokines. A commercial BDTM Cytometric Bead Array Mouse Th1/Th2/Th17 Cytokine Kit (CBA) (lot: 77184, BD Biosciences, San Jose, CA, USA) was used to determine the levels (pg/ml) of TNF-α in serum samples, according to the manufacturer's instructions. Fluorescence was analyzed using a flow cytometer (BDTM LSR II flow cytometer system; BD Biosciences) and the cytokine level was determined using a BD CBA Software (BD Biosciences).

Western blot analysis. Western blot analysis was performed as previously described.[8] Immunoblotting was performed using the following antibodies: anti IκB-α and IκB-β (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-BCL-2 (Santa Cruz Biotechnology) and anti-Caspase-3 (Sigma-Aldrich, St Louis, MO, USA). The antibodies were used according to standard procedures. Equal protein loading of blots was confirmed by immunoblotting of glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Santa Cruz Biotechnology). Densitometry of the bands was obtained by chemi DOC XRS+ image lab software (Bio-Rad Laboratories, Hercules, CA, USA). Results are presented as fold of change over control (untreated WT group), which is defined as 1.

Real-time quantitative PCR. RNA extraction was performed with Tri Reagent (Sigma-Aldrich) according to the manufacturer's instruction. cDNA was reverse transcribed from total RNA with random primers using the High-Capacity cDNA Reverse Transcription Kit with RNase inhibitor according to the manufacturer's instruction (Applied Biosystems, Foster City, CA, USA). mRNA expression was quantified by TaqMan (Applied Biosystems) using the Illumina high-performance Eco Real-Time PCR system. Quantitative real-time PCR (TaqMan) assays were conducted in triplicates, and standard deviation was used to calculate error bars.
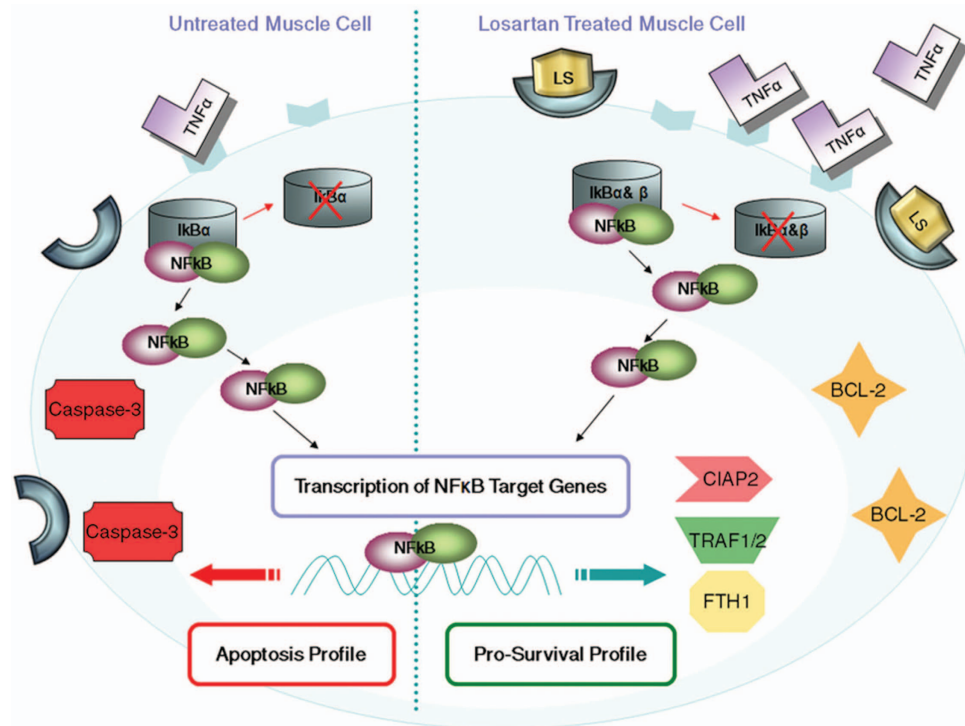
Figure 6 Proposed model for NFκB as a key regulator in the survival path of muscle following Losartan treatment. Losartan treatment increases TNF-α, which in turn activates NFκB through IκB-α and IκB-β degradation, p65 nuclear accumulation and upregulation of the pro-survival genes: *TRAF1, TRAF2, CIAP2*, and *FTH1* in addition to anti-apoptotic BCL-2 protein, to mediate the anti-apoptotic effect of NFκB in skeletal muscle of the dy^{2J}/dy^{2J} mouse model of MDC1A. In addition, Losartan treatment results in decreased expression of the pro-apoptotic protein Caspase-3

Immunofluorescence. Quadriceps muscles were isolated and fixed in acetone for 1 h. The quadriceps muscle tissues were sliced into 8 μm cross sections after being embedded in OCT. The mounted sections were washed three times in phosphate-buffered saline (PBS), blocked with 1% bovine serum albumin and probed with anti-p65 rabbit antibody (Abcam, Cambridge, UK) and with anti-dystrophin mouse antibody (Santa Cruz Biotechnology). After three washes in PBS, the sections were incubated with Alexa fluor 647-conjugated affini-pure donkey anti-rabbit IgG (H+L) and with Cy2-conjugated affini-pure donkey anti-mouse IgG (H+L) (Jackson ImmunoResearch, West Grove, PA, USA). After three more washes in PBS, coverslips were mounted on glass slides with a DAPI-containing mounting medium (Vector Laboratories, Burlingame, CA, USA). Fluorescence analysis was performed using a Zeiss LSM 710 confocal laser scanning system (Carl Zeiss MicroImaging GmbH, Jena, Germany). The P65-positive fluorescent cells were counted under a fluorescent microscope, and the numbers were expressed as the percentage of total P65 area cells ± S.D. A negative control without anti-p65 rabbit antibody (Abcam) was also performed.

Tunel staining. Quadriceps muscles sections were assessed by terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assay with an *In Situ* Cell Death Detection Kit (Roche Diagnostic, Indianapolis, IN, USA). Muscles were isolated and fixed in acetone for 1 h; the tissues were sliced into 8 μm cross sections after being embedded in OCT. Afterwards washed twice in PBS. Each slice was embedded with 100 μl permeabilisation solution, 0.1% Triton X-100 for 2 min, washed twice in PBS and the tissue sections were labeled and stained with the TUNEL reaction mixture (label plus enzyme solutions) for 60 min at room temperature and washed twice with PBS. Then the slices were probed with anti-dystrophin mouse antibody (Santa Cruz Biotechnology), and after three more washes in PBS the sections were incubated with Alexa fluor 647-conjugated affini-pure donkey anti-mouse IgG (H+L) (Jackson ImmunoResearch). After three more washes in PBS, coverslips were mounted on glass slides with a DAPI-containing mounting medium (Vector Laboratories). The apoptotic fluorescent cells were counted under a fluorescent microscope, and the numbers were expressed as the percentage of total Tunel area cells ± S.D. A negative control without enzyme treatment and a positive control with DNase I (Roche Diagnostic) treatment were also performed.

Statistical analysis. All data are expressed as mean and standard error of the mean. Statistical analysis for direct comparison between two groups was performed by unpaired Student's *t* test. Significance was set at $P < 0.05$ for all comparisons.

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